UNIVERSITY OF LIVERPOOL

Adult epithelial stem cells in the human endometrium: existence, location and 3D histo-architecture

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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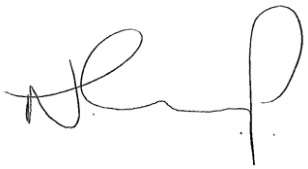
Declaration

I declare that the work presented in this thesis is all my own work and has not been submitted for any other degree.



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Abbreviations

ASCs	Adult stem cells
α-SMA	Alpha smooth muscle actin
AR	Androgen receptor
APES	3-Aminopropyltriethoxysilane
BDT	Big Dye Terminator
BrdU	Bromodeoxyuridine
CE	Cloning efficiency
CFU	Colony forming units
CBC	Crypt base columnar
Ct	Cycle threshold
CCO	Cytochrome C oxidase
DCIS	Ductal carcinoma in situ
EC	Endothelial cell
ESC	Embryonic stem cells
EGF	Epidermal growth factor
EpCAM	Epithelial cell adhesion molecule
EVT	Extravillous trophoblast
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FACS	Fluorescence activated cell sorting
FSH	Follicle stimulating hormone
FOXA2	Forkhead Box A2
FFPE	Formalin fixed paraffin embedded

GE	Glandular epithelia
HSP	Heat-shock promoter
H&E	Hematoxylin and Eosin
IF	Immunofluorescence
IHC	Immunohistochemistry
iPSCs	Induced pluripotent stem cells
ICM	Inner cell mass
ISH	*In situ* hybridisation
IVF	*In vitro* fertilisation
ISK	Ishikawa cells
Ki67-LI	Ki67-labelling index
LRCs	Label retaining cells
LCM	Laser capture micro dissection
LMP	Last menstrual period
LGR5	Leucine-rich repeat-containing G-protein-coupled receptor 5
LIF	Leukaemia Inhibitory factor
LNG-IUS	Levonorgestrel-releasing intrauterine system
LE	Luminal epithelium
LH	Leuteinising hormone
MACS	Magnetic Cell Sorting
MPA	Medroxyprogesterone acetate
Tm	Melting temperature
MSC	Mesenchymal stem cells
mRNA	messenger RNA

mtDNA Mitochondrial DNA

Msi1 Musashi1

Msi2 Musashi2

NBF Neutral-buffered formalin

NSG NOD scid gamma mice

ER Oestrogen receptor

ERα Oestrogen receptor alpha

PBS Phosphate buffered saline

PDGF-Rβ Platelet derived growth factor–receptor β

PODXL Podocalyxin-like protein 1

PCR Polymerase chain reaction

PM Post-menopausal

POP Progesterone only pill

PUGKO Progesterone-induced uterine gland knockout

PR progesterone receptor

PI Propodium iodide

qRT-PCR Quantitative reverse transcription- polymerase chain reaction

ROS Reactive oxygen species

RT-PCR Reverse transcriptase- polymerase chain reaction

rRNA Ribosomal RNA

RSPO1 R-Spondin 1

RT Room temperature

SEM Scanning electron microscope

SP Side population

SSCs	Somatic stem cells
SOX9	SRY-Box 9
SSEA-1	Stage specific embryonic antigen -1
3D	Three dimensional
TSH	Thyroid stimulating hormone
TNAP	Tissue Non-specific Alkaline Phosphatase
TFs	Transcription factors
tRNA	Transfer RNA
TA	Transient amplifiers
2D	Two dimensional
UV	Ultraviolet
UGKO	Uterine gland knockout

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Abstract

The human endometrium is a highly regenerative organ undergoing over 400 cycles of shedding and regeneration over a woman's life time. Menstrual shedding and subsequent repair of the endometrial functionalis layer is a process unique to humans and higher-order primates. This massive regenerative capacity is thought to have a stem cell basis, with the human endometrial stromal stem cells already widely studied. However, studies on endometrial epithelial stem cells are sparse, and the current dogma is that the endometrial epithelial stem cells reside in the terminal ends of the basalis glands at the endometrial/myometrial interface. Consequently, there is a lack of information available regarding endometrial epithelial stem cells, and the histo-architecture of the endometrial epithelial compartment. Since almost all endometrial pathologies are thought to be originating from aberrations in stem cells that regularly regenerate the functionalis layer, expansion of our current understanding of stem cells is necessary in order for curative treatment strategies to be developed. The principal goal of the work presented in this thesis was to determine if the human endometrial epithelium has a stem cell that is likely to be involved in glandular regeneration, and to understand the three-dimensional histo-architecture of the endometrial epithelial organisation.

There are many stem cell markers described in different tissues using extensive characterisation and functional confirmatory studies. Evidence from work in the intestine, hair follicles and kidneys showed Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) to be an epithelial stem cell marker. In the initial part of the work in this thesis, the expression of *LGR5* in the human endometrium was examined to ascertain if it is likely to be a marker of endometrial epithelial stem cells. This was accomplished by confirming the expression, and subsequently examining the cellular localisation of *LGR5* in all epithelial compartments of the human endometrium, and the Fallopian tube. The hormone regulation of *LGR5* in the endometrium was also studied *in vitro* and *in vivo*. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was initially used to

determine the presence of *LGR5* in the endometrium and Fallopian tube across the menstrual cycle, showing higher expression in the oestrogen dominant proliferative phase of the cycle. The gold standard method for locating *LGR5* expression at a cellular level, *in situ* hybridisation (ISH), was in agreement with the qRT-PCR data regarding the differences of *LGR5* expression across the menstrual cycle, but, surprisingly, the location of *LGR5* expressing cells was not limited to the postulated stem cell niche, the basalis glands. Our data demonstrated the luminal epithelium (LE) to have significantly higher levels of *LGR5* expression than all other epithelial compartments in the endometrium; basalis glands also constitutively expressed *LGR5* albeit at lower levels than LE. When *LGR5* expression was considered in the context of the expression of the previously known progenitor markers SSEA-1 and SOX9, the luminal and basalis epithelia shared distinct, region specific, patterns of co-expression. Levels of *LGR5*, SSEA-1 and SOX9 decreased in all 3 endometrial epithelial compartments, and also in the tubal epithelium in the secretory phase, when compared with the samples from the proliferative phase, suggesting a hormonal (progesterone) regulation. To further investigate the possible regulation of *LGR5* by progesterone, endometrial explants, treated with progesterone *in vitro* (medroxyprogesterone acetate (MPA)) and *in vivo (*patients on exogenous progestagenic treatment*)*, were examined, demonstrating a decrease in *LGR5* expression. *From this work we hypothesised that it is possible for the human endometrium to have more than one epithelial stem/progenitor cell pool*.

The current consensus had been that the endometrial glandular arrangement is similar to the intestinal glandular crypts, assuming a single blunt ended ductular design. Utilising a three dimensional (3D) reconstruction technique, previously used in the liver and kidney, we created three 3D endometrial reconstructions. These reconstructions revealed that the premenopausal functionalis glands are non-branching and run a vertical and parallel course through the superficial functionalis layer of the human endometrium. The basalis glandular arrangement was very different to this, and formed a complex branching configuration proceeding in a horizontal course to the

myometrium. *This novel finding, basalis assuming a root-like "stolon" configuration, is likely to provide an effective glandular propagation strategy to maintain continuous endometrial covering of the uterine cavity, and if the stem cells are residing in them, contribute efficiently to the scar-less regeneration of the endometrium.*

With the unexpected findings in the *LGR5* study and the newly discovered 3D histo-architecture of the endometrial epithelium, we required direct evidence as to the existence of endometrial epithelial stems cells. Therefore, in the final part of this thesis, *in vivo* lineage tracing, the "gold standard" method of stem cell identification, was utilised to prove the existence of endometrial epithelial stem cells for the first time. Tracing non-pathogenic mitochondrial DNA (mtDNA) mutations using cytochrome C oxidase (CCO)-deficiency as a marker, is a natural experiment allowing lineages to be traced, and stem cell niches to be identified. The amount of CCO-deficient clonal patches, were found to be increased in incidence across the reproductive years, up to the age of menopause, with the earliest patch seen in the basalis of a 25 year old patient. This suggested the long lived epithelial cells, (likely stem cells), to be located in the basalis. Using single cell Sanger sequencing of the whole mitochondrial genome following laser capture micro dissection (LCM), a unique mtDNA mutation was found in all CCO-deficient cells, of a partially mutated gland, and absent in all adjacent CCO-proficient wild type cells, conclusively confirming the existence of endometrial epithelial stem cells in the endometrium. The existence of partially mutated glands, suggests that at least some endometrial glands are regenerated by more than one epithelial stem cell (polyclonal origin). The clonal patches were found to contain markers specific to all three endometrial epithelial compartments, suggesting the different cell subtypes comprising the human endometrial glandular compartment has a common ancestral origin, i.e. a multipotent epithelial stem cell. Using a 3D model of CCO staining, the extent of the clonal patches was examined. The clonal population, extending to the functionalis glands, suggested that they had risen from stem cells located in the basalis glands. *The work presented in this thesis therefore describes for the first time, that the human endometrium harbours unique mtDNA*

mutations, proving the existence of endometrial epithelial stem cells, and the endometrial glandular regeneration to be polyclonal. Although the method only allows for a snapshot in time, the technique does demonstrate the two major aspects of stemness, multipotentiality and self-renewal.

In conclusion, the work described in this thesis presents three novel findings: *LGR5* expression in the human endometrial epithelium has presented a new conundrum regarding the location of the stem cell niche in the human endometrial epithelium; the 3D reconstruction of the endometrial epithelium has shown non-branching vertical and parallel functionalis glands and intricate branching horizontal basalis glands forming a root-like “stolon” architecture; utilising non-pathogenic mtDNA mutations, and lineage tracing, the existence of endometrial epithelial stem cells has been proven for the first time. This work therefore provides a novel basis for further work on the exact location, dynamics, and changes in diseased tissue of stem cells, and discovering their identity in the future.

Chapter 1. General Introduction

Human endometrium, which lines the uterine cavity, is the main target for ovarian steroid hormones. During the reproductive period of a woman's life, the endometrium undergoes up to 400 monthly cycles of proliferation and sloughing under the influence of the ovarian hormonal signals. Since tissue regeneration is directly dependent on adult stem cell (ASC) function, the remarkable regenerative ability of the endometrium, seen during this monthly loss and repair cycle, should also have a stem cell basis. The work undertaken in this thesis, examines the stem cell involvement in normal endometrial epithelial regeneration, and the histo-anatomical architecture of the endometrial epithelial compartment.

1.1 Development of the uterus

1.1.1 Early fetal development of the human uterus

The sperm, at fertilisation, determines the genetic sex of an embryo, but before 7 weeks, the genital systems are similar in both sexes; the *indifferent or primitive stage* of the reproductive organs (Edgar, Mazor et al. 2013). In the female embryo, development of the uterus and tubes occurs, because of the absence of a Y chromosome, and lack of testosterone from testicular tissue, not because of the presence of an ovary, (Jost, Vigier et al. 1973). This is also the case if no gonad is present. In the seventh week, the paramesonephric (Müllerian) ducts arise from the mesoderm, lateral to the mesonephric ducts, as focal invaginations of the coelomic epithelium, on the upper pole of each mesonephros (Healey 2012, Edgar, Mazor et al. 2013). The paramesonephric ducts, are the precursors of the uterus, Fallopian tubes, cervix, and upper vagina, growing caudally, coursing lateral to the urogenital ridges (Healey 2012). The cranial end of the fused ducts yields the single median uterovaginal primordium; the future uterus. The epithelium, and glands of the uterus (Healey 2012), the endometrial stroma, and myometrium, form from adjacent mesenchyme (Edgar, Mazor et al. 2013). The unfused cranial ends of the paramesonephric ducts assume a funnel

shaped configuration, and remain open to the future peritoneal cavity as the fimbrial portions of the Fallopian tubes (Healey 2012, Edgar, Mazor et al. 2013). The caudal end of the fused ducts will form the upper two thirds of the vagina (Healey 2012). Lateral fusion of the paramesonephric ducts occurs between the seventh and ninth weeks, when the lower segments of the paramesonephric ducts fuse. At this stage, a midline septum is present in the uterine cavity, which usually regresses at around 20 weeks, but can persist even postnatal (Healey 2012). Simultaneously, the mesonephric (Wolffian) ducts regress, and any abnormality that occurs during this phase of development may result in a variety of paramesonephric anomalies in women.

1.1.2 Overview of the uterus

The adult human uterus is a hollow muscular organ, approximately 7.5 cm in length (Figure 1.1). It serves to act as a conduit for the migrating sperm, and houses the developing embryo and foetus. In terms of tissue remodelling, it is arguably the most plastic organ in a woman, expanding up to 1000 times in volume and 20 fold in weight during pregnancy, and returning to near pre-pregnancy size after parturition (Ono and Maruyama 2015).

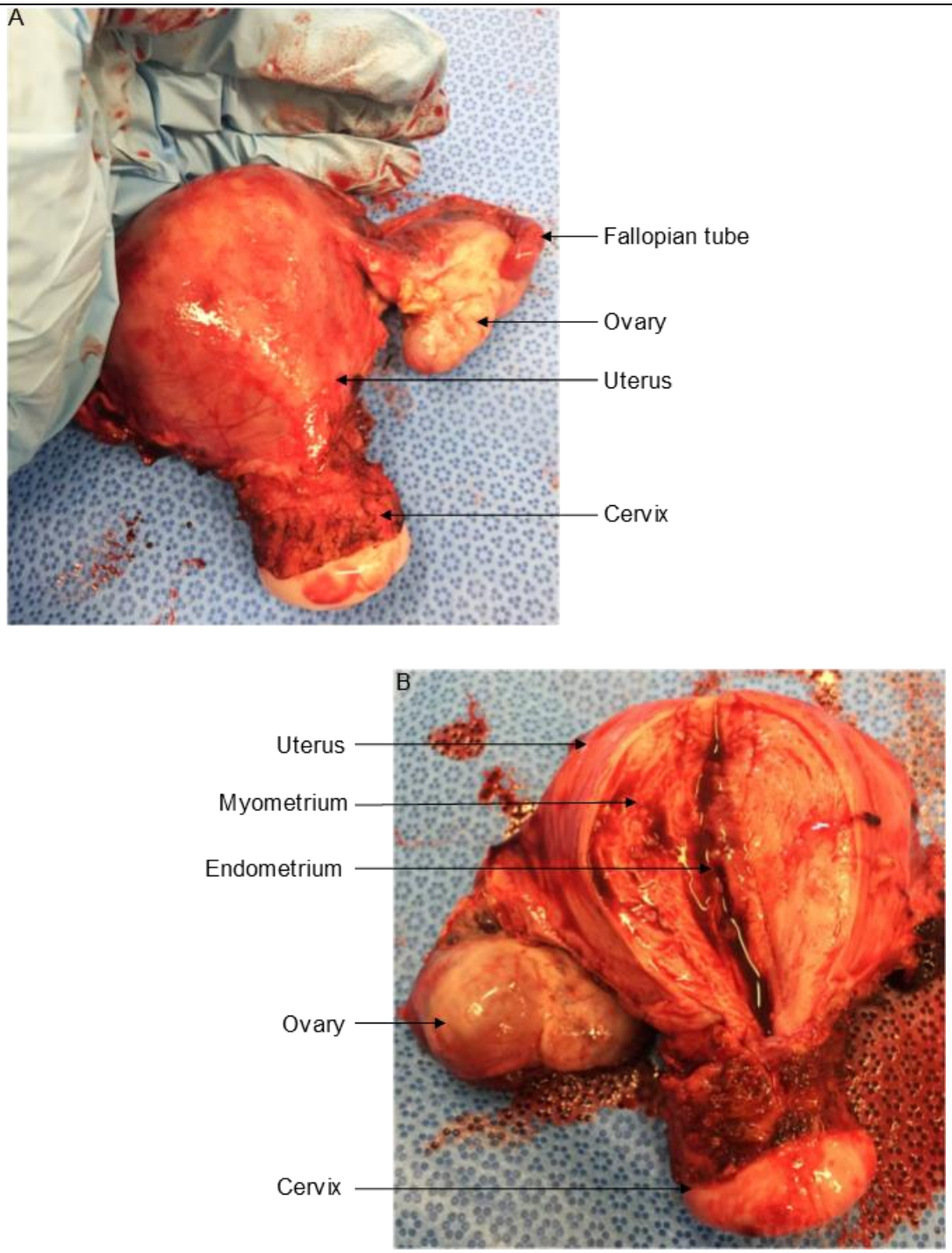


Figure 1.1 – Photograph of a human uterus following hysterectomy and bilateral salpingo-ophrectomy. (A) Labelled clinical sample (B) Labelled clinical sample following opening of the anterior aspect of the uterus in the sagittal plane.

Most human women have one uterus; however, sometimes there appears to be double uteri because of the incomplete development in utero. This is called a Mullerian Anomaly. There are many uterine anomaly variants, ranging from a uterine septum, to uterine didelphys (double uterus) (Sosa-Stanley and Bhimji 2017) (Figure 1.2).

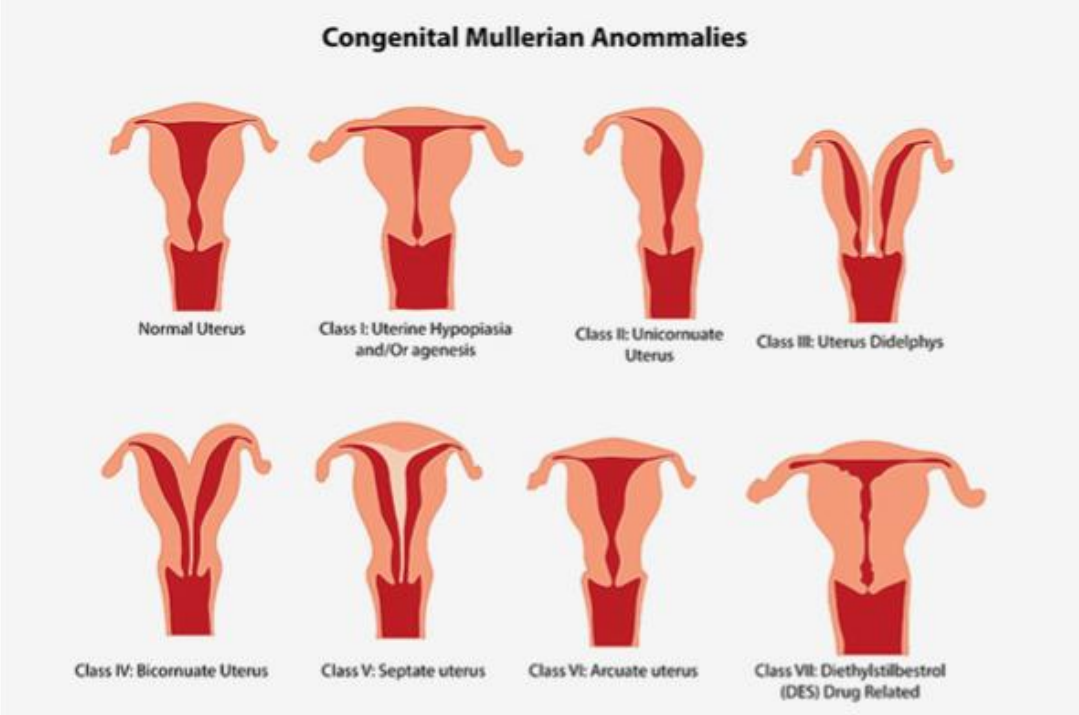


Figure 1.2 – Illustrations of congenital Mullerian anomalies.

The uterus consists of the fundus, the corpus, and the cervix, with three openings to the uterine cavity; the two Fallopian tubes and the external os (connecting the uterus with the vagina and the outside environment). The uterus is covered with peritoneum and the uterine wall contains a thick muscular outer layer (myometrium), a criss-cross of involuntary fibres mixed with fibro-elastic connective tissue, and an inner lining, the endometrium (Figure 1.3).

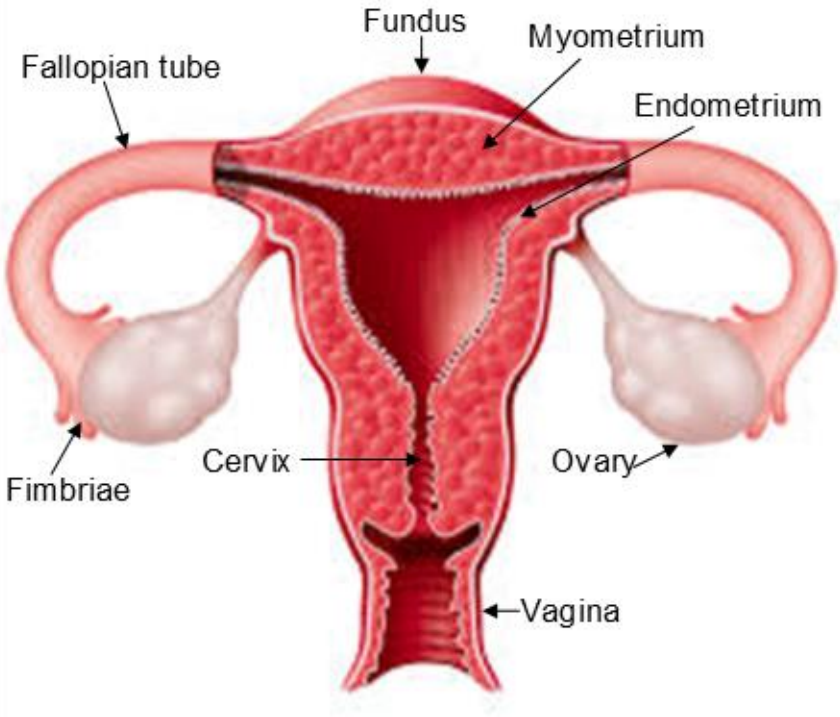


Figure 3 – Figure of the female reproductive organs.

The uterus is typically located, in the female pelvis, posterior to the bladder and anterior to the rectum, supported by several ligaments, including the utero ovarian ligament, round ligament, broad ligament, cardinal ligament, and uterosacral ligaments (Figure 1.4). It is further supported (inferiorly) by the pelvic diaphragm, urogenital diaphragm, and perineal body.

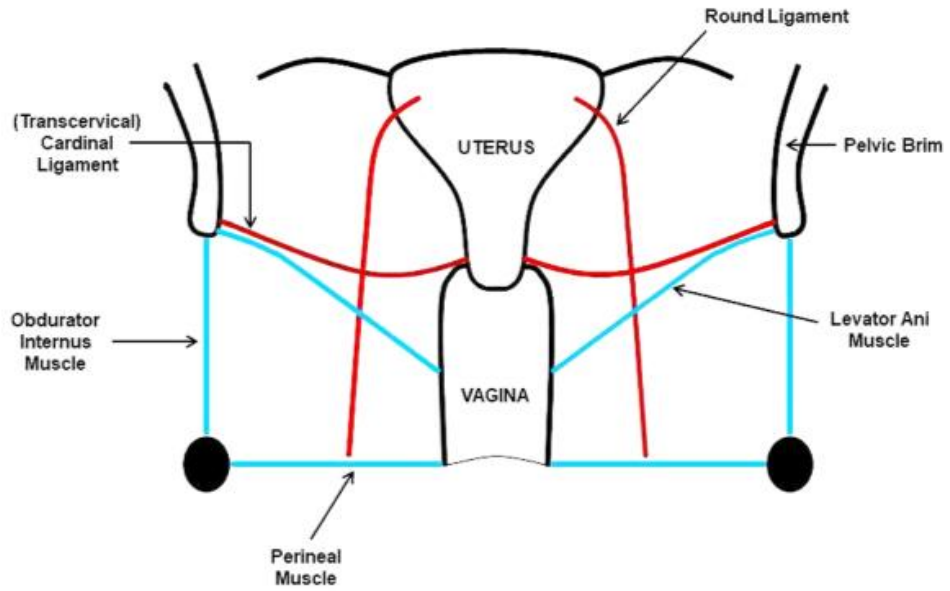


Figure 1.4 – Schematic of the uterine ligament support network.

The uterus may naturally lie in different positions such as ante-verted/retro-verted, ante-flexed/retro-flexed, or midline, and it may be rotated (especially during pregnancy). In 50% of women, the uterus lies in an ante-flexed and ante-verted position (Sosa-Stanley and Bhimji 2017) (Figure 1.5).

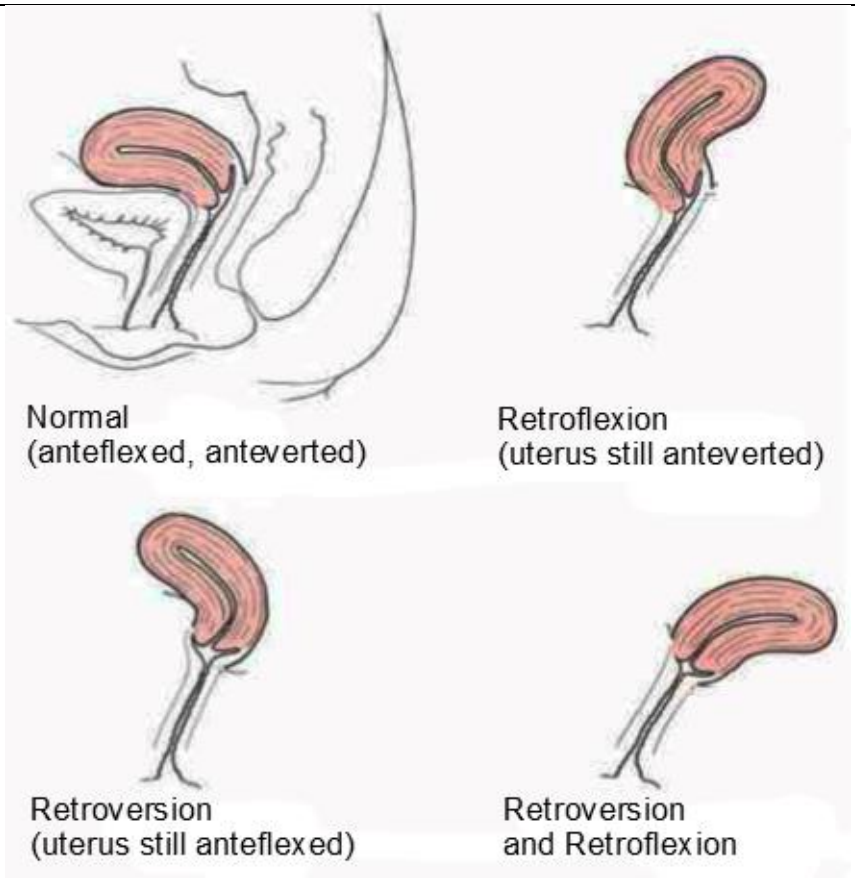


Figure 1.5 - Illustrations of uterine positions in the pelvis.

The uterus receives a blood supply from the uterine and ovarian arteries, which arise from the anterior branch of the internal iliac artery. As the blood supply enters the myometrium, it branches into the arcuate arteries, which branch into the radial arteries. As they enter the level of the endometrium, they branch into the basal and spiral arteries (Sosa-Stanley and Bhimji 2017).

Nerves from T11 and T12 innervate the uterus; the sympathetic supply is from the hypogastric plexus, and the parasympathetic supply is from S2 to S4.

1.2 **The Endometrium**

Endometrium is the complex, innermost mucosal layer of the uterus. It consists of 2 main resident specific cell types; stromal and epithelial cells; it also contains blood vessels, leucocytes, nerves and lymphatic vessels.

1.2.1 Endometrial Gland development

While in utero (as in rodents and ungulates), uterine glands begin to develop in the female fetus as invaginations of luminal epithelium (LE), giving rise to primordial glandular epithelial buds (Gray, Bartol et al. 2001, Cooke, Spencer et al. 2013). By 20–22 weeks of gestation, the myometrium is well developed, but adeno-genesis (gland-formation) is superficial (Song 1964). Scattered glands that exist are straight, narrow, and consist of a pseudostratified appearance to the epithelium (Wang 1989). On the apical surface of the LE, microvilli, cilia, and cytoplasmic projections can be seen (Wang 1989). As the gestation progresses, by the 7^{th} and 8^{th} months, more glands are seen, but the pseudostratified configuration of the epithelium persists (Wang 1989). By the 9^{th} gestational month, glands become slightly tortuous and dilated, where simple columnar epithelium predominates, and a pseudo-stratification arrangement is seen only rarely. The microvilli, cilia, and cytoplasmic projections become increasingly common in the LE (Wang 1989). In addition to this, in the 9^{th} gestational month, scanning electron microscopy (SEM) studies have found isolated triangular, or elongated cells, containing pale cytoplasm and numerous, highly electron dense, granules (approximately 0.3 to 0.8 µm in diameter) at the base of the epithelium, together with some less electron dense granules, grouped in the basal region, with the same morphology as endocrine cells (Wang 1989). The exact function and further characterisation of these cells has not, as yet, been determined. At late gestation, the fetal endometrial glands display a morphological resemblance to the proliferative phase endometrial glands of a sexually mature women (Wang 1989).

During the pre-pubertal period, from birth to puberty, the glands progressively invade the mesenchyme with extensive tubular coiling and branching morphogenesis (Gray, Bartol et al. 2001) resulting in an extensive network of epithelial glands throughout the stroma (Cooke, Spencer et al. 2013) extending to the myometrium. The glands extend half way to the myometrium by the age of 6 years (Valdes-Dapena 1973, Gray, Bartol et al. 2001).

1.2.2 Overview of the endometrium

Histologically, the adult human and primate endometrium is divided into two functionally different layers. The upper functionalis; which contains glands loosely held together by supportive stroma, and the lower basalis; consisting of branching glands and dense stroma (Ferenczy and Bergeron 1991, Gray, Bartol et al. 2001) (Figure 1.6A).

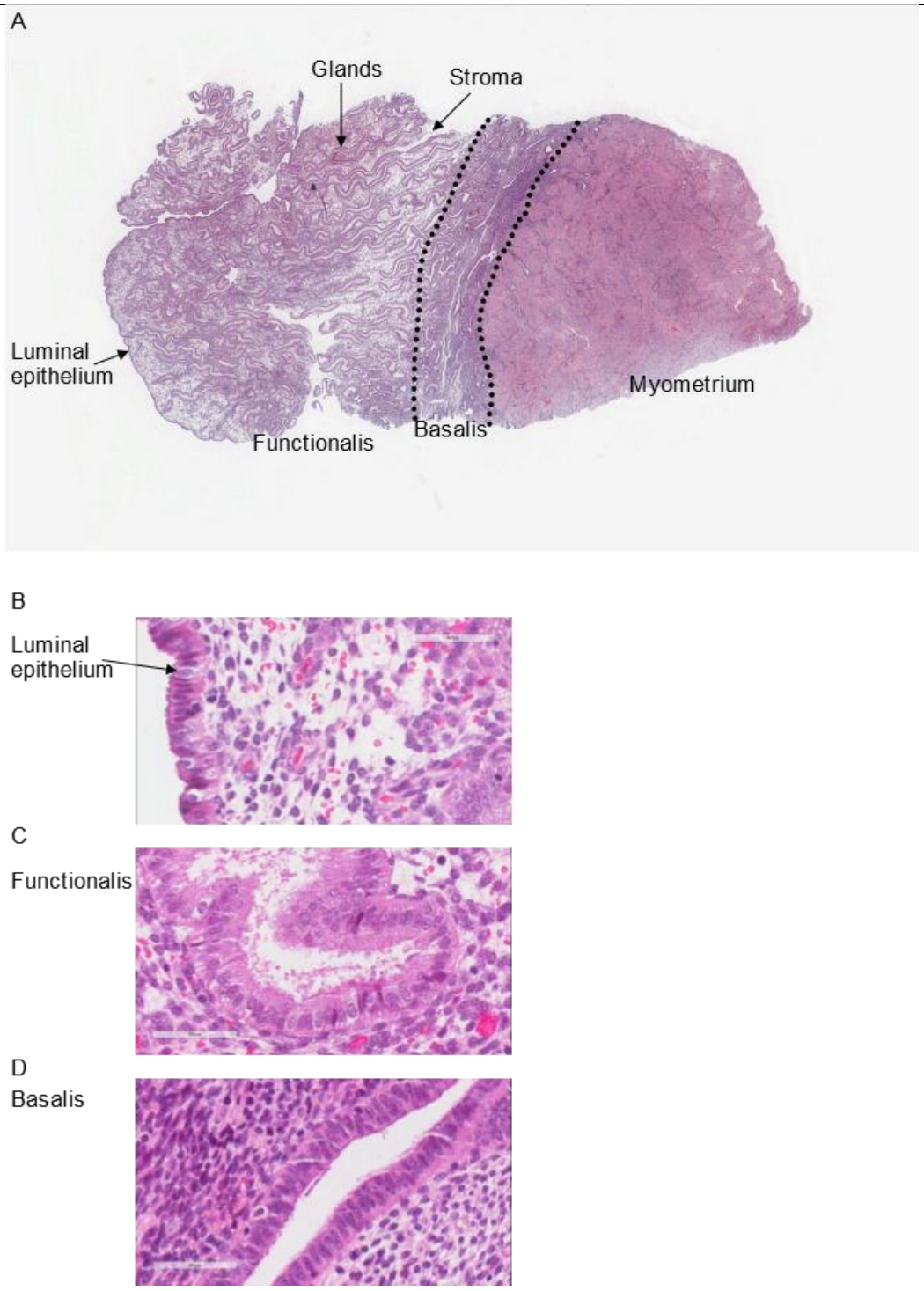


Figure 1.6 – The layers of the human endometrium. (A) All layers of the human endometrium stained with Hematoxylin and Eosin (H&E). (B) Representative micrograph of the LE (magnification x400). (C) Representative micrograph of the functionalis epithelium (magnification x400). (D) Representative micrograph of the basalis epithelium (magnification x400).

1.2.2.1 The luminal epithelium

The LE is a single layer of cuboidal epithelial cells lining the functionalis epithelium. It is supported by stromal fibroblasts (Evans, Martinez-Conejero et al. 2014), and invaginates into the underlying cellular stroma to form tube-like glands (Figure 1.6B). LE cells are less abundant than their glandular counterparts and, therefore, could be underrepresented in studies that used either whole endometrial tissue, or isolated epithelial cells (Evans, Martinez-Conejero et al. 2014).

Ultra-structurally, LE cells have distinctive euchromatic nuclei with prominent nucleoli, and relatively loose cell membranes in both cellular poles (apical and basal) (Demir, Kayisli et al. 2002). In the follicular phase of the cycle, LE possess many ciliated cells (Nilsson, Englund et al. 1980), located near gland orifices, at tubal corners, and close to the endo-cervical mucosa (Baraggino, Dalla Pria et al. 1980). The number of cilia varies from cell to cell and includes an average of 60 branches; each with a length of 8 µm, and width of 0.2 µm, with growth in relation to oestrogenic stimulus (Baraggino, Dalla Pria et al. 1980). Their role is to remove the secretion products of the bordering cells, and take an active part in the kinetics of the spermatozoa, and in the capitation of the oocyte. Ciliated to non-ciliated cells are present at a ratio of 1:30 during the 5^{th} to 6^{th} day of the cycle, but this gradually increases in the pre-ovulatory period (Baraggino, Dalla Pria et al. 1980). The non-ciliated cells carry numerous, fairly long microvilli; a number of these have an irregular surface with small apical protrusions (Nilsson, Englund et al. 1980), (hairy branches) (Baraggino, Dalla Pria et al. 1980). In some areas, they are arranged to form what looks like cobbled paving intersected by deep grooves, with the presence of a greater number of microvilli in the oestrogen driven proliferative phase of the cycle, compared with the secretory phase (Baraggino, Dalla Pria et al. 1980). The apocrine secretory activity of non-ciliated cells is proved by the presence of round apical prominences with a 2-4 µm diameter, wrinkled and umbilicated surface. These cells are irregularly distributed and increased in number during the 20^{th} to 24^{th} day (“window of implantation”) of the menstrual cycle (Baraggino, Dalla Pria et al. 1980). The LE and glandular epithelia (GE) are distinct from

each other, despite both potentially originating from the basalis. The LE cells are cuboidal, unlike the columnar shaped GE (Aplin 2006). This fundamental difference in shape, thus creates other variables such as differences in the degree of secretions, changes to the internal structure (van Mourik, Macklon et al. 2009), variations in location, and also in their threshold of response to ovarian steroids (Bartlemez G 1951).

The LE is the first maternal layer of cells that an embryo communicates with during the window of implantation (Gray, Bartol et al. 2001), thus, it is central to determining the receptivity of the endometrium (Evans, Martinez-Conejero et al. 2014). LE undergoes apical surface specialisation, expressing cell adhesion molecules that permit adherence of the blastocyst (Bruce Lessey 2013), and controls excessive trophoblastic invasion (Tabibzadeh and Babaknia 1995). Therefore, molecular expression at the LE determines the early steps of embryo attachment and implantation, and if the mRNA and protein expression of these cells is not appropriate, implantation may not be successful (Evans, Martinez-Conejero et al. 2014). In 2014, laser capture micro dissection (LCM) of LE and GE from endometrial tissue (and subsequent microarray and immunohistochemistry (IHC) experiments) was used to show that, in the same sample, expression of many genes and proteins in the LE were different to that of GE. These findings indicate that different cellular processes may occur in LE and GE, despite them existing as a continuous monolayer (Evans, Martinez-Conejero et al. 2014). Fifty of 242 down-regulated LE genes were upregulated in the GE, and 34 of 159 genes were down-regulated in GE, but were up-regulated in LE. Of the 84 significantly and contradictorily regulated genes, 18 were involved in catalytic activity (transferase and hydrolase), and 23 were involved in binding (protein and nucleic acid), pathways involved in metabolism. IHC was used to further confirm the findings of the gene expression study, demonstrating, in all samples examined, stronger staining of the LE than the GE for androgen receptor (AR), oestrogen receptor α (ERα), cyclin-dependent kinase Inhibitor 2A, Mouse double minute 2 homolog, matrix metalloproteinase-7, matrix metalloproteinase-11, and oviductal glycoprotein 1. Interestingly, the aforementioned stronger LE staining of AR and ERα had a noticeable

reduction in staining where the LE monolayer invaginated to form a gland. This work reiterates how important it is to treat the LE as a distinct entity to the GE (Evans, Martinez-Conejero et al. 2014).

A recent preliminary study further reported that CK5/6 protein expression, and lack of Podocalyxin-like protein 1 (PODXL) marked the LE cells. Further identification of cell surface markers, with expression limited to the LE or GE such as PODXL, may provide the opportunity to isolate these cells, and to further examine the freshly-isolated cells for their phenotypical, as well as functional properties, rather than using LCM which only allows phenotypical characterisation (Maclean A 2017). However, such isolation methods also have drawbacks, since the mechanical and enzymatic digestion that is required for releasing cells from the solid organ (endometrium) may affect the gene expression, and cells, when they are removed from their natural three dimensional (3D) environment, they may demonstrate different functional properties to their normal, physiological behaviour.

1.2.2.2 The functionalis epithelium

In numerous studies, the endometrial functionalis and basalis were examined without splitting into separate entities and they were loosely termed as the GE. When the GE ultrastructure is considered, there are mainly two types of cells; dark and clear cells (Demir, Kayisli et al. 2002). The functionalis epithelia proliferate massively in response to oestrogen and differentiate in response to progesterone; changing their features throughout the menstrual cycle, ready to assist with conception; accepting the invading trophoblast. When compared with the relatively less active basalis layer, most of the cyclical changes observed in the cellular phenotype (gene/protein expression; changes in cell fate of the endometrium) occur in this layer (Bruce Lessey 2013) (Figure 1.6C).

During the early secretory phase, the glandular cells have a huge accumulation of glycogen particles, giant mitochondria, and the occurrence of a nucleolar channel system. As the secretory phase proceeds, apocrine secretions and giant lysosomes become the striking findings (Cornillie, Lauweryns et al. 1985). The glands have been described to be vertically

orientated, perpendicular to the LE, with relatively large diameters, substantial lumens and irregular outer borders (Garry, Hart et al. 2010).

The decidual cells in the functionalis, and large granular lymphocytes, modulate the trophoblast function, and endometrial angiogenesis, through the following secretions: growth factors; growth factor binding proteins; angiogenic factors; cytokines (Bruce Lessey 2013, Filant and Spencer 2013). The GE and their secretions play a fundamental role during early pregnancy by secreting substances that support blastocyst development (Bruce Lessey 2013); its absence being associated with reduced conceptus survival (Gray, Bartol et al. 2001). As maternal circulation to the human placenta is not established until 10-12 weeks gestation, GE helps to sustain the trophoblast during the first-trimester. Research using a sheep model of uterine gland knockout (UGKO) emphasised the importance of GE and secretions for conceptus survival and implantation (Gray, Taylor et al. 2001, Gray, Burghardt et al. 2002). Deficient glandular activity during the secretory phase of the cycle in humans has been correlated with unexplained infertility (Dimitriadis, Stoikos et al. 2006, Burton, Jauniaux et al. 2007). Filant et al demonstrated the important role of GE in supporting successful mouse implantation by utilising the progesterone-induced uterine gland knockout (PUGKO) mouse model (neonatal mice are exposed to progesterone from postnatal day 2 to 10 which permanently ablates the differentiation of uterine glands, the adult mice cycle normally, but are infertile) to test the hypothesis that uterine glands and their secretions are essential for blastocyst implantation, and stromal cell decidualisation, in the uterus. The PUGKO mice do not contain GE but do contain LE, stroma and myometrium, and no implantation occurs in the mice but blastocysts are made. Other researchers have shown that the lack of Leukaemia Inhibitory factor (LIF) leads to implantation, and decidualisation defects, and this is also secreted from the GE (Stewart, Kaspar et al. 1992). A further recent study found that conditional ablation of Forkhead Box A2 (*FOXA2*) in the postnatal mouse uterus using a PGR-Cre mouse model, resulted in a reduction, and near absence of endometrial glands in the adult. Thus indicating, *FOXA2* is a critical regulator of endometrial adenogenesis in the mouse uterus, and also

demonstrating that GE are required for implantation, as these mice blastocysts did not implant (Jeong, Kwak et al. 2010). The results from the three sets of mouse work (aglandular PUGKO; *FOXA2* mutant; LIF null mice) support the need for GE, and their secretions, for blastocyst implantation, and stromal cell decidualisation, both of which are required for successful pregnancy in mice and humans (Filant and Spencer 2013). Accordingly, an increased understanding of uterine gland biology is important for diagnosis, prevention, and treatment of fertility, and pregnancy problems, in mammals (Spencer 2014).

Furthermore, thin endometrium is a well-recognised factor in subfertility and *in vitro* fertilisation (IVF) failure, and is obviously associated with inadequate glandular growth, but its diagnosis and treatment are controversial. The current literature does not justify the use of measuring the endometrial thickness to decide on IVF cycle cancellation, freezing of embryos, or refraining from further IVF (Kasius, Smit et al. 2014). Autologous cell therapy with $C133^{+}$ bone marrow derived stem cells, combined with simultaneous hormone replacement therapy for refractory Asherman`s syndrome, and endometrial atrophy has been attempted (Santamaria, Cabanillas et al. 2016). The authors report that in the first three months, the therapy increased the volume, and duration, of menses as well as the endometrial thickness, and angiogenesis processes of the endometrium, while decreasing intrauterine adhesion scores (patients diagnosed with Asherman`s syndrome were classified according to the American Fertility Society classification of uterine adhesions (Society 1988)) However, the apparent benefit was short lived, with patients` scores returning to baseline within 6 months post-completion of the therapy (Santamaria, Cabanillas et al. 2016). Treatment for thin endometrium remains a challenge, and future large research studies are required to further elucidate and optimise management of these patients (Lebovitz and Orvieto 2014).

1.2.2.3 The basalis epithelium

Similar to the functionalis, the endometrial basalis is also dynamic. The functionalis and basalis are morphologically similar, both with high nucleus-

to-cytoplasm ratios and elongated sausage-shaped nuclei, with dense chromatin and inconspicuous nucleoli; the nuclei appear pseudostratified, especially when proliferating (Hendrickson 2007). However, the basalis is also the structurally stable and permanent compartment of the uterus, not eroded during menstruation or at parturition (at the end of gestation), this tissue is, therefore, postulated to function as the germinal compartment of the human endometrium (Gray, Bartol et al. 2001, Valentijn, Palial et al. 2013). The basalis rests on the myometrium, contains fewer glands and denser stroma, and is present throughout a woman's life (Figure 1.6D). The glands respond weakly, at best, to hormones, and the stroma is inactive, therefore, the basalis should not be used to morphologically date endometrium (Hendrickson 2007). Since this layer is retained after menstrual shedding of the functionalis, the stem/progenitor cells are postulated to reside in the basalis (Prianishnikov 1978, Gargett, Chan et al. 2007). The endometrial ablative procedures that are used to treat excessive menstruation, therefore, are designed to destroy this deeper layer, and also to abolish the stem cells that reside there, with the expectation that complete cessation of menstruation due to lack of subsequent regeneration will happen. Nevertheless, this is not the case in a number of women, and even after extensive iatrogenic destructive procedures (Tresserra, Grases et al. 1999) the endometrium regrows in some women (range 25-75% of women having endometrial ablation), who will continue to bleed (Gimpelson 2014, Muller I 2015).

The basalis glands are less responsive to hormones than the functionalis glands; hence, they do not undergo rapid change throughout the menstrual cycle (Slayden and Keator 2007, Bruce Lessey 2013). Surface markers have been used to differentiate the basalis epithelium from the functionalis, namely nuclear β-catenin, nuclear SRY-Box 9 (SOX9), surface Stage specific embryonic antigen -1 (SSEA-1) (Valentijn, Palial et al. 2013) and recently, surface marker N-cadherin (Nguyen, Xiao et al. 2017). Both nuclear β-catenin and nuclear SOX9 suggest activation of the Wnt signalling pathway in these cells, and this is an important pathway for adenogenesis in the endometrium and in the intestine (Valentijn, Palial et al. 2013).

1.2.2.4 The blood supply of the endometrium

The endometrial vasculature is supplied by the arcuate arteries of the adjacent myometrium, giving rise to radial arteries, which traverse through the myometrium to reach the basalis as basal arteries, and spiral arteries which supply the functionalis, and are drained by their corresponding veins (Ramsey 1989). Uterine blood vessels are comprised of outer layers of vascular smooth muscle cells, and extracellular matrix, with an inner endothelial cell layer (Biswas Shivhare, Bulmer et al. 2018). Although basal arteries are not hormonally responsive, the spiral arteries are exceedingly hormonally responsive. Markee showed that in a rhesus monkey model of intraocular endometrial menstruation, following hormone withdrawal, the coiled arteries constricted, terminating the haemorrhage from them (Markee 1946).

Angiogenesis is one of the main features of the endometrium. Normal physiological angiogenesis occurs in most organs during fetal growth and development, but it is a rare occurrence in adult organs, except during episodes of wound healing (regeneration) and in the female reproductive tract. Throughout the reproductive life of females, angiogenesis occurs regularly in the corpus luteum and the endometrium, as part of the rapid growth and regression that occurs in these tissues during the menstrual cycle (Gargett and Rogers 2001, Demir, Yaba et al. 2010).

During the menstrual cycle, angiogenesis is spatially and temporally regulated under the control of oestrogen and progesterone (Rogers, Donoghue et al. 2009, Demir, Yaba et al. 2010) with; (A) vascular repair in the superficial layers of the remaining basalis in the menstrual phase (Maybin and Critchley 2015); (B) angiogenesis throughout the functionalis of the proliferative phase, supporting rapid endometrial growth; and (C) growth and coiling of the spiral arterioles in the secretory phase (Rogers, Donoghue et al. 2009, Biswas Shivhare, Bulmer et al. 2018) in the functionalis (Demir, Yaba et al. 2010). The capillary plexus, supplied by arterioles, develops just below the LE surface. Vascular endothelial growth factor, messenger RNA (mRNA), and protein are present during all phases in the endometrium, but

its expression increases during the late secretory phase, and menstruation (Chen, Liu et al. 2015).

Spiral artery remodelling has 2 phases: a decidua-associated, or extravillous trophoblast (EVT) independent phase; and an EVT-dependent phase (Lash, Pitman et al. 2016). The importance of angiogenesis for endometrial function and epithelial growth is demonstrated by many studies (Alfer, Happel et al. 2017). Remodelling of the spiral arteries is a key maternal adaptation required for successful human pregnancy, failure being associated with pre-eclampsia, fetal growth restriction, and miscarriage (Robson, Harris et al. 2012, Lash, Pitman et al. 2016). `Sub-fertile` patients with abnormally thin endometrium showed adequate serum concentrations of hormones, but a severe deficiency of angiogenesis-related marker molecules, leading to a defectively thin endometrium, potentially providing an explanation for the endometrial factor causing infertility (Alfer, Happel et al. 2017). A major side effect of progesterone only contraceptives is also abnormal uterine bleeding; this is again caused by problems with the endometrial vasculature. Women display abnormally enlarged, fragile blood vessels with decreased endometrial blood flow and oxidative stress, this abnormal bleeding leads to discontinuation of use of said contraception by many women (Smith and Critchley 2005, Hickey, Krikun et al. 2006, Lockwood 2011, Guzeloglu Kayisli, Kayisli et al. 2015). Heavy menstrual bleeding is also related to aberrant endometrial angiogenesis, with stromal cell-derived factor 1 and endothelial cells being disturbed (Kooy, Taylor et al. 1996, Elkilani and Soliman 2017), affecting a woman’s quality of life and requiring medications and surgery. Potential anti angiogenic medications are being assessed (Rae, Mohamad et al. 2009).

1.2.2.5 The nerves of the endometrium

The available studies on the endometrial nerve supply are contradictory, regarding the depth in the endometrium they reach, their density, and if the amount/density changes according to the menstrual cycle, or with disease. Studies have reported that nerves vary greatly from patient to patient in both their number, and the level to which they penetrate the endometrium, and

these variations do not seem to correlate with the phase of the cycle or pathology (Dallenbach and Vonderlin 1973). The variation, potentially, seems to correlate with the degree of menstrual shedding of a patient (Dallenbach and Vonderlin 1973). Straight coursing bundles of nerves of variable sizes have been found to reach the endometrium from the myometrium, often without following blood vessels. In the basalis, the nerve bundles branch into smaller bundles, or into single fibres, with a high density of fine nerve trunks and axons (Miller and Fraser 2015), these are normally distributed (Ellett, Readman et al. 2015) and often assume a wavy tortuous course. At the functionalis they pass near glands and, it could be assumed that, the level at which nerve fibres are found in the endometrium, approximates to the level at which shedding occurs to at menstruation (Dallenbach and Vonderlin 1973). Uterine nerve fibres degenerate with the progression of pregnancy and regenerate after parturition (Zoubina, Fan et al. 1998). These nerves are suggested to be involved in endometriosis associated pain, as well as aberrant nerve fibre expression being a diagnostic feature of the disease (Tokushige, Markham et al. 2006, Tokushige, Markham et al. 2007). These claims, however, were not substantiated by further studies (Ellett, Readman et al. 2015).

1.2.2.6 The lymphatics of the endometrium

The lymphatic vasculature functions to maintain fluid haemostasis within tissues, and to direct and regulate immunological responses in the body (Swartz, Hubbell et al. 2008). Lymphatic vessels have been identified in all layers of the endometrium, but with a significant reduction of vessels in the functionalis, where they are small and sparsely distributed compared with the basalis (Donoghue, Lederman et al. 2007), which are larger and closely associated with spiral arterioles (Donoghue, Lederman et al. 2007, Rogers, Donoghue et al. 2009). The observation that lymph fluid, returning from the superficial endometrium, comes into intimate contact with the smooth muscle cells in the wall of the spiral arteries, opens up the possibility of a novel mechanism of regulation for these specialised vessels. Any factors secreted by either the endometrium or the implanting embryo, will have direct access to the spiral artery wall via the lymphatic’s (Rogers, Donoghue et al. 2009,

Volchek, Girling et al. 2010). Patients with recurrent pregnancy loss, show compromised extra villous trophoblast invasion of the venous and lymphatic vasculature, highlighting the importance of this access to the lymphatics (Windsperger, Dekan et al. 2017).

1.2.3 The endometrial cycle

In non-pregnant, pre-menopausal women, oestrogen is primarily released from the granulosa cells of the ovary, under the regulation of the hypothalamic-pituitary-ovarian axis (Figure 1.7). Oestrogen is a primary sex hormone that regulates endometrial regeneration (Kamal, Tempest et al. 2016). Relatively smaller amounts of oestrogen are also produced by extra-ovarian tissue, such as adipose tissue, liver, and adrenal glands (Kamal 2016). There are three forms of endogenous oestrogen: oestradiol (E2, the most potent oestrogen) predominates in reproductive life, estrone (E1, the weaker oestrogen) predominates after menopause, and the third is oestriol (E3, the least potent) produced by the placenta (Kamal 2016, Kamal, Tempest et al. 2016). Progesterone is the second major hormone involved in the endometrial cycle and is essential for cellular differentiation, it is a steroid hormone secreted by the ovarian corpus luteum that develops after ovulation in women. Progesterone promotes decidualisation, counteracts oestrogen-induced proliferation, and, if conception occurs, maintains the pregnancy (Kamal, Tempest et al. 2016). Adrenals also produce progesterone, which is largely converted into glucocorticoids and androgens, without being released in to the circulation. The half-life of progesterone is as short as five minutes, being either promptly deactivated in the liver or converted in the kidney to a potent mineralocorticoid (Taraborrelli 2015, Kamal, Tempest et al. 2016).

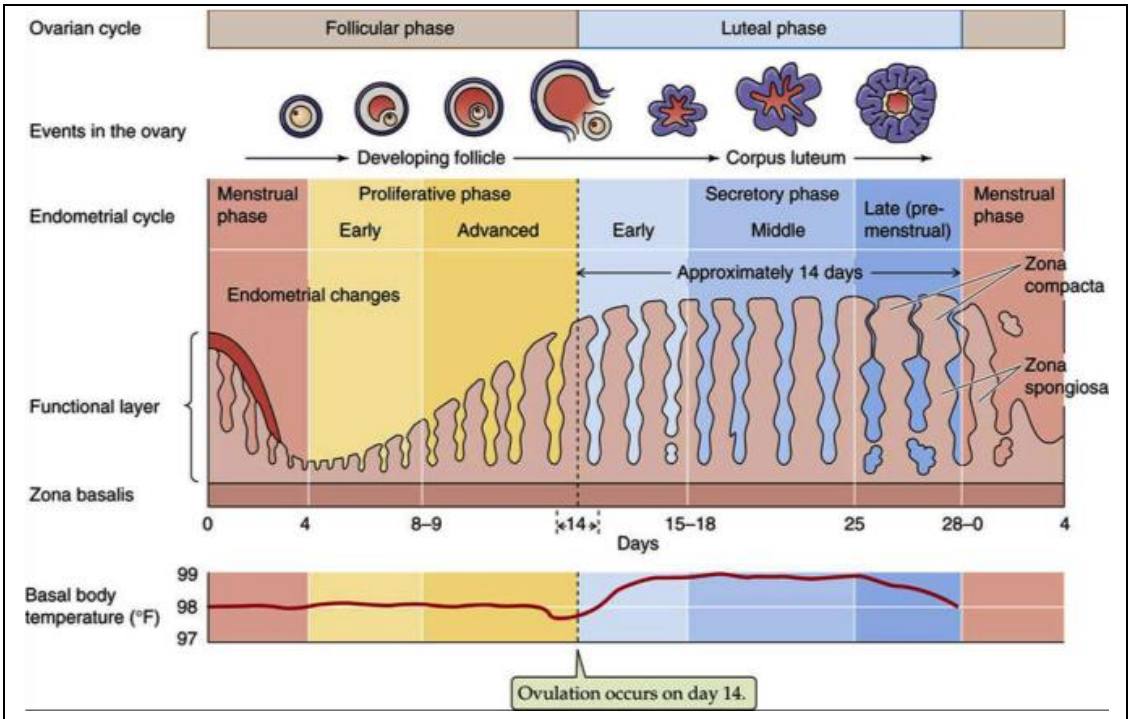


Figure 1.7 – The ovarian and endometrial cycle.

1.2.3.1 The menstrual phase of the endometrial cycle

In the absence of implantation, the degeneration of the corpus luteum lowers the amount of circulating oestradiol and progesterone, and this, in turn, triggers the cascade of events resulting in the shedding of the endometrial functionalis. As menstruation proceeds, and regeneration commences, the surface of the endometrium is rapidly re-epithelialised by epithelial cells. This is completed within 48 hours post shedding, and a new cycle then commences (Sturgis SH 1936, Chan, Schwab et al. 2004). The most accepted theory to date suggests that the regenerating surface epithelium originates from the residual epithelium of the basal gland stumps (Dallenbach-Hellweg 2012). However, others have proposed different theories including; the transformation of endometrial stromal cells into regenerating surface epithelial cells (Baggish, Pauerstein et al. 1967); regeneration of new epithelial cells by the transformation of peritoneal cells after their migration through the oviducts (Singer, Reid et al. 1968).

Experiments in non-menstruating species, such as rats and rabbits, have shown that the earliest signs of repair (as evidenced by the presence of flattened epithelial cells covering part of the denuded surfaces) occur 3 hours after surgical curettage (Schenker, Sacks et al. 1971). Therefore, a similar, rapid repair process is expected to happen in the human endometrium.

SEM studies have revealed a shared origin for reepithelialisation. Outgrowth from exposed basal glands ran alongside ingrowth from the peripheral surface membrane; importantly, these studies have shown the stromal regeneration to only commence once the denuded endometrium has been completely re-epithelialised (Ferenczy 1976).

Examination of the isthmic and corneal uterine mucosal segments revealed foci of stromal haemorrhage and inflammatory exudate, but the endometrial architecture, including the surface epithelium, remained intact following menstruation (Ferenczy 1976), this may suggest the possibility of the intact surface epithelium from these regions contributing to the re-epithelialisation of the denuded endometrial surface during the regenerative process. On the third menstrual day, there was an irregular ragged surface present because of the menstrual shedding; most of the basal zone was denuded, with a few foci of the residual spongy layer. The basalis showed no disintegration of stroma and glands, merely shrinkage (Dallenbach-Hellweg Gisela 2010). SEM studies showed that many basal gland crypts were orientated parallel to the long axis of the uterine cavity, with their body and neck arranged obliquely or perpendicular to the surface into which opened haphazardly spaced gland mouths (Ferenczy 1976).

Surface epithelium of the isthmic and cornual origin establish anastomosis with peripheral portions of the LE, originating in the gland stumps of the zona basalis (Ferenczy 1976). Both the basal glands and the peripheral uterine regions remain intact during the entire menstrual period (Ferenczy 1976).

The endometrial surface regeneration is likely a local reaction to injury, induced by loss of endometrial mucosa, rather than being mediated by ovarian hormones, as morphologic changes known to be associated with oestrogenic stimulation, only occur following complete surface re-

epithelialisation, coinciding with the increase in plasma and tissue receptor levels of oestradiol-17 beta (Ferenczy 1976, Ferenczy 1976). The lack of ERα receptor, which mediates the classical mitotic effects of oestrogen in endometrial primitive cell populations, may also agree with this observation (Hapangama, Kamal et al. 2015). The aberrations in endometrial regeneration are obviously likely to cause clinical manifestations, such as heavy menstrual bleeding, and researchers are actively seeking to enhance current knowledge on the endometrial regenerative process, exploring novel avenues to treat millions of women worldwide suffering with this common gynaecological condition.

1.2.3.2 The proliferative phase of the endometrial cycle

The proliferative phase can be of variable length (10-20 days) but the suggested ideal duration is 14 days (Deligdisch 2000). Growth of the new endometrial functionalis (intensive mitosis in the GE and in the stroma) begins in this phase in response to the rising levels of circulating oestrogen (Deligdisch 2000). The endometrium at this phase is reported to be thin, with sparse, narrow, and straight glands evenly distributed in a loose stroma of spindle-shaped cells. The GE cells are low, columnar, and contain small rounded or oval shaped chromatin dense nuclei in a scanty cytoplasm. The stromal cells are poorly differentiated and of equal size with small, dense nuclei in a scanty cytoplasm. Nucleoli are inconspicuous and mitoses are rare. The cells are surrounded by a firm reticulin network and the surface epithelium is flat and still regenerating (Dallenbach-Hellweg Gisela 2010). Endometrial thickness proceeds to increase more than 10 fold (Deligdisch 2000, Dallenbach-Hellweg Gisela 2010), glands assume a simple tube-like shape, with narrow lumens, lined with columnar epithelial cells (Gray, Bartol et al. 2001). By the late proliferative phase, they become longer and more tortuous (Gray, Bartol et al. 2001); the spiral arteries wind themselves lightly into the stroma with the functionalis stroma becoming more oedematous (Ferenczy and Bergeron 1991). The oestrogen-induced changes are mediated via ER. The presence of nuclear ERs in endometrial cells are responsible for the translation of hormonal signals into structural changes (Deligdisch 2000).

GE cells, in particular in the proliferative phase, were reported to be tall and columnar, and contain slightly enlarged oval-shaped, chromatin-rich nuclei in a dense, sparse cytoplasm, rich in RNA (Dallenbach-Hellweg Gisela 2010). They were covered with long microvilli demonstrating a well-developed glycolcalyx layer (Gray, Bartol et al. 2001). Changes observed at an ultra-structural level include a marked increase in the number of rough endoplasmic reticulum and Golgi apparatus (Cornillie, Lauweryns et al. 1985), protein synthesis by free ribosomes, and rough endoplasmic reticulum, as well as accumulation of intermediate filaments, mitochondria (spherical, elongated and irregular (Cornillie, Lauweryns et al. 1985)), and lysosomes. The nuclei increased in size; nucleoli became prominent; and chromatin became coarse. Individual cell mass and nuclear: cytoplasmic ratio also increased, with numerous mitoses seen throughout the glands and stroma (Deligdisch 2000, Gray, Bartol et al. 2001, Dallenbach-Hellweg Gisela 2010) and lipid droplets observed (Cornillie, Lauweryns et al. 1985, Gray, Bartol et al. 2001). The spindle-shaped stromal cells were still poorly differentiated, but they were rich in DNA and RNA with occasional mitoses, and were separated by interstitial oedema (Dallenbach-Hellweg Gisela 2010).

When ovulation is imminent (in the mid-cycle), there are large round mitochondria associated with semi-rough endoplasmic reticulum. In non-dividing glandular cells, the nucleus usually exhibited a euchromatin texture suggesting a high level of transcription activity. Massive cellular accumulation of glycogen in the GE was a hallmark of the late proliferative, and early secretory phases (Cornillie, Lauweryns et al. 1985).

At the end of the proliferative phase the oestradiol peak (released by the growing follicles) triggers a positive feedback mechanism at the level of the pituitary, and ovulation commences 35 to 55 hours after the initial luteinising hormone (LH) increase (Choi and Smitz 2014).

1.2.3.3 The secretory phase of the endometrial cycle

Rising progesterone (from the corpus luteum) during the secretory phase, blocks epithelial mitoses, inhibiting proliferative activity (Deligdisch 2000),

and cells commence differentiation. Stromal proliferation reduces, and the stroma features vacuoles and granulocytes, predecidualisation begins around blood vessels and extends to the stroma beneath the surface epithelium. Numerous basal glycogen vacuoles appear in the GE (in, at least, 50% of the glandular cells), pushing the nucleus towards the lumen (Dallenbach-Hellweg Gisela 2010). The glands dilate and are lined by low columnar epithelium. By the late secretory phase, they are irregular in shape with large lumens, and are lined by tall columnar epithelial cells (Garry, Hart et al. 2010), allowing endometrial cells to undergo secretory differentiation with the subsequent exposure to progesterone that is released by the post-ovulatory corpus luteum. The secretory changes only take place in an oestrogen-primed endometrium (Deligdisch 2000).

At an ultrastructure level, in the early secretory phase, glandular cell nuclei demonstrated a euchromatin pattern with only chromatin condensation along the nuclear membrane. Nucleoli were well developed alongside a fully differentiated nuclear channel system (Gray, Bartol et al. 2001). Glycogen particles were found in the glandular lumen amidst other amorphous secretory material. Mitochondria were numerous and well developed (Gray, Bartol et al. 2001). The rough endoplasmic reticulum, Golgi apparatus, and glycogen stores of the endometrial stromal cells became well developed. These changes contributed to the predecidual transformation of the stromal cells, which progressively became separated by accumulation of oedematous fluid, while the number of mononuclear phagocytes, lymphocytes, and endometrial granulocytes gradually increased towards the end of the cycle (Cornillie, Lauweryns et al. 1985).

In the mid secretory phase, the nucleus retained the basal position with larger amounts of heterochromatin. A well-developed labyrinth of rough endoplasmic reticulum cisternae remained throughout the cytoplasm, but focal dilatation was less pronounced. Glycogen particles were present in basal paranuclear, and apical cytoplasm. Glycogen was found in the glandular lumen, together with other glycoprotein secretory material (Gray, Bartol et al. 2001).

Lipid droplets became apparent in the subnuclear cytoplasm in association with decreasing stores of glycogen (Gray, Bartol et al. 2001). In the late secretory period the nucleoli were less conspicuous and glycogen remained to be present in small sub and paranuclear patches, larger amounts were found in the supranuclear cytoplasm and in apical blebs protruding into glandular lumen, with intraluminal glycogen as an additional feature. The mitochondria were numerous and small, and the Golgi system was also very prominent (Gray, Bartol et al. 2001).

If pregnancy does not occur, endometrial glands regress late in the secretory phase, commensurate with luteolysis, and break down during menstruation (Gray, Bartol et al. 2001). The main function of the endometrium, embryo implantation, occurs in the secretory phase, which makes it the most important cycle phase. Researchers therefore focus on the changes associated with the many endometrial pathologies, in this phase.

1.2.3.4 Implantation

The proposed time period of the maximal receptivity for the blastocyst in a 28 day cycle is between the 20th and 23rd day. This 4 day time frame is termed the window of implantation. By day 20, flattened ‘droplet like’ projections appear on the luminal surface called pinopodes/uterodomes, and they are believed to be ‘landing platforms’ for the potential blastocyst; they disappear within 4 days of formation.

Muc-1, a mucin thought to cause steric hindrance between the blastocyst and luminal apical surfaces, is locally down-regulated at implantation sites (Surveyor, Gendler et al. 1995, Meseguer, Aplin et al. 2001). With the expression of αvβ3 vitronectin receptor integrin on both LE and GE coinciding with the time of embryo attachment, aberrant expression of this integrin has been found to be associated with infertility (Lessey, Ilesanmi et al. 1996).

Endometrial progesterone receptor (PR) is critical for embryo implantation (Mulac-Jericevic and Conneely 2004), yet the loss of PR expression in the uterine epithelium is considered as a prerequisite for implantation (Bazer and

Slayden 2008). The expression of PR in the LE is sustained when local oedema is present at the implantation site and disappears from the LE, not before implantation, but in the middle of the implantation process (Diao, Paria et al. 2011).

If implantation occurs, endometrial stromal cells complete their decidualisation and will be terminally differentiated; this is thought to apply to the epithelium as well. Most fertility researchers focus their studies to this phase of the cycle, for obvious reasons.

1.3 **The Fallopian tubes**

The Fallopian tubes develop from the cranial parts of the paramesonephric ducts, with their cranial ends remaining open connecting the duct with the coelomic (peritoneal) cavity, and the caudal end communicating with the uterine cornua (El-Mowafi DM 2017).

The human Fallopian tubes are paired tubular, sero-muscular organs, approximately 10-15 cm long and 6-8 mm in diameter with one end open into the peritoneal cavity (near the ovary) (Edgar, Mazor et al. 2013) and the other into the superior lateral part of the uterine cavity (El-Mowafi DM 2017). The tubes are situated in the upper margins of the broad ligaments, between the round and utero ovarian ligaments. The tube conducts the ova, discharged from the primordial follicle at ovulation, in to the uterine cavity, and can be broken into 4 different regions for descriptive purposes (Figure 1.8): the infundibulum is funnel shaped and formed of a number of fimbria; the ampulla is the largest section and thin walled like the infundibulum; the isthmus is short and smaller in diameter but thicker walled than the ampulla; the interstitial portion is the segment embedded in the uterine wall with a small diameter of 1 mm (El-Mowafi DM 2017).

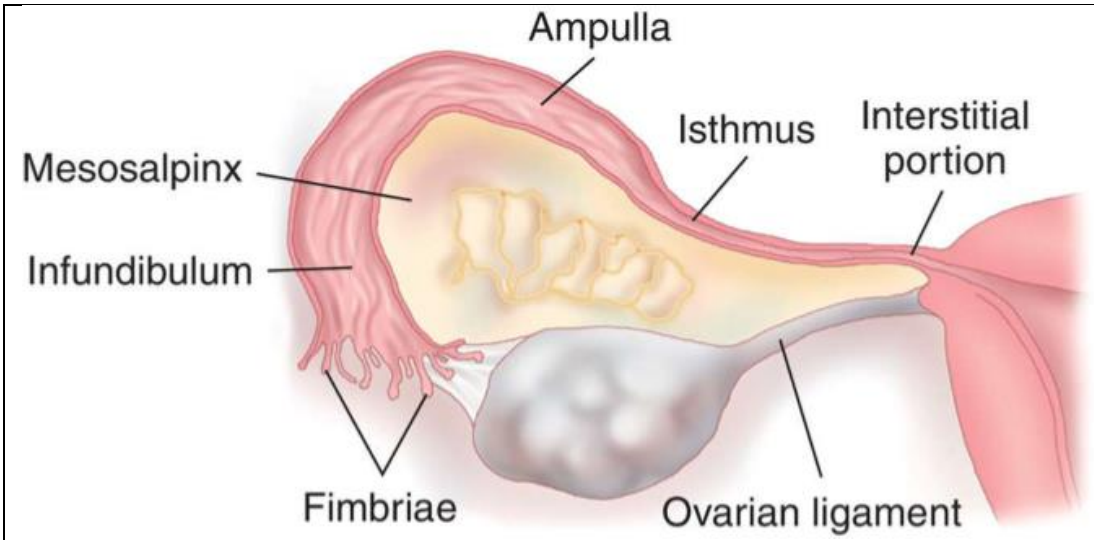


Figure 1.8 - Illustration of the human Fallopian tube.

The Fallopian tube wall consists of a series of layers, namely: the internal mucosa (endosalpinx); the muscularis (myosalpinx); and the serosa. The internal mucosa is thrown into longitudinal folds (primary folds) increasing in numbers towards the fimbria and lined by columnar epithelium of three types: ciliated cells; secretory cells; and peg cells. Ciliated cells are characterised by pale stained nuclei and long, slender cilia protruding into the tubal lumen (Novak E 1928) with progressively increasing numbers from the proximal to distal end of the Fallopian tube (Crow, Amso et al. 1994). These cells can be identified by β-tubulin and TUBB4 expression (Paik, Janzen et al. 2012). Secretory cells overhang ciliated cells, and are narrower, containing secretory granules in their apical regions (Woodruff JD 1969). They are found at higher numbers in the ampulla (Paik, Janzen et al. 2012), and are marked by PAX8 expression (Paik, Janzen et al. 2012). Peg cells are rarer and interspersed between ciliated and secretory cells (Paik, Janzen et al. 2012), they are usually identified by the expression of EPCAM, CD44 and integrin 6 (Paik, Janzen et al. 2012). The three cell types of the Fallopian tube have distinct markers, making it possible to identify and examine cell specific function. The peg cells, are fivefold enriched for cells capable of clonal growth and self-renewal, thus are thought to be the stem cells (Paik, Janzen et al. 2012). Unlike the Fallopian tube, similar information is lacking for the different epithelial cell types of the endometrium epithelium.

In the ampullary and infundibular sections, secondary folds of the tubal mucosa exist, increasing the surface area. The internal mucosa secretes mucous, in order to maintain the ovums journey through the Fallopian tube, and it also undergoes cyclical epithelial changes. The height and proportion of ciliated to non-ciliated secretory cells vary; the epithelium is reported to be taller in the first half of the follicular phase, and the relative number of non-ciliated cells increased in the secretory phase. However, in comparison to the endometrium, there is no organised and cyclical shedding in this mucosal layer. The muscularis was thickest at the isthmus, and thinned toward the fimbrial end. It had a well-developed inner circular layer, and thin outer longitudinal layer (complete only at the isthmus). The longitudinal muscle bundles that are present were discontinuous in the ampulla and may be absent in the fimbria (Ernst LM 2011). The outer serosal layer has the structure of peritoneum, and is continuous with the peritoneum of the broad ligament and uterus, the upper margin of which is the mesosalpinx (El-Mowafi DM 2017). Although the available literature on the exact hormone responsiveness of the Fallopian tube mucosa is sparse, tubal epithelium shares the same embryological origin; continues from the endometrium; and expresses ovarian hormone receptors. Yet, a cycle of similar growth, shedding, and regeneration does not happen in the tube; and why this is so, remains to be a mystery.

1.3.1.1 Blood, nerve and lymphatic supply of the Fallopian tubes

Blood supply to the Fallopian tube is derived from the tubal branch of the uterine artery, and small branches from the ovarian artery. Generally, branches of the uterine artery supply the isthmus and proximal ampulla, while branches of the ovarian artery supply the remainder of the tube. The arteries run in the stroma along the bases of the folds, giving rise to a dense capillary stromal network, with the veins coursing a similar way (Eddy and Pauerstein 1980).

The autonomic innervation of the tube is sympathetic and parasympathetic. The distribution pattern of extrinsic tubal adrenergic nerves is composed of long post ganglionic fibres spreading out distally from the hypogastric, celiac

and pelvic ganglia of short neurons, originating in the proximal ganglia located in the cervicovaginal region. The afferent innervations carrying pain sensation are derived from T11 and T12, and upper lumbar nerves. The parasympathetic supply is dual; the distal portion of the tube is supplied by vagal fibres and from the ovarian plexus, and the sacral parasympathetic fibres are derived from S2,3,4 and are conveyed to the terminal ganglia of the pelvic plexuses. Short postganglionic fibres from this plexus supply the interstitial portion of the isthmus (Eddy and Pauerstein 1980).

The lymphatic's follow the blood vessels and drain into the lumbar (or aortic) nodes (Eddy and Pauerstein 1980, Edgar, Mazor et al. 2013). Three separate lymphatic networks drain the mucosa, muscularis, and serosa (Eddy and Pauerstein 1980).

1.4 Stem cells

1.4.1 History

Stem cells are undifferentiated cells, without any tissue-specific function, that have the potential to differentiate into certain tissues determined by their genetic and epigenetic program (Nemeth and Karpati 2014). The term “stem cell” first appeared in scientific literature as early as 1868, coming from the German “Stammzelle” or “family cell”. Ernst Haeckel, a phylogenist, used the term to describe a single cell, from which all multicellular organisms were believed to originate. Although controversial at the time, the concept was later echoed by Russian histopathologist Alexander Maksimow, and applied to the haematopoietic system in 1905. It took another 50 years for the theory to be replaced by fact, as a consequence of the pioneering work by Till and McCulloch.

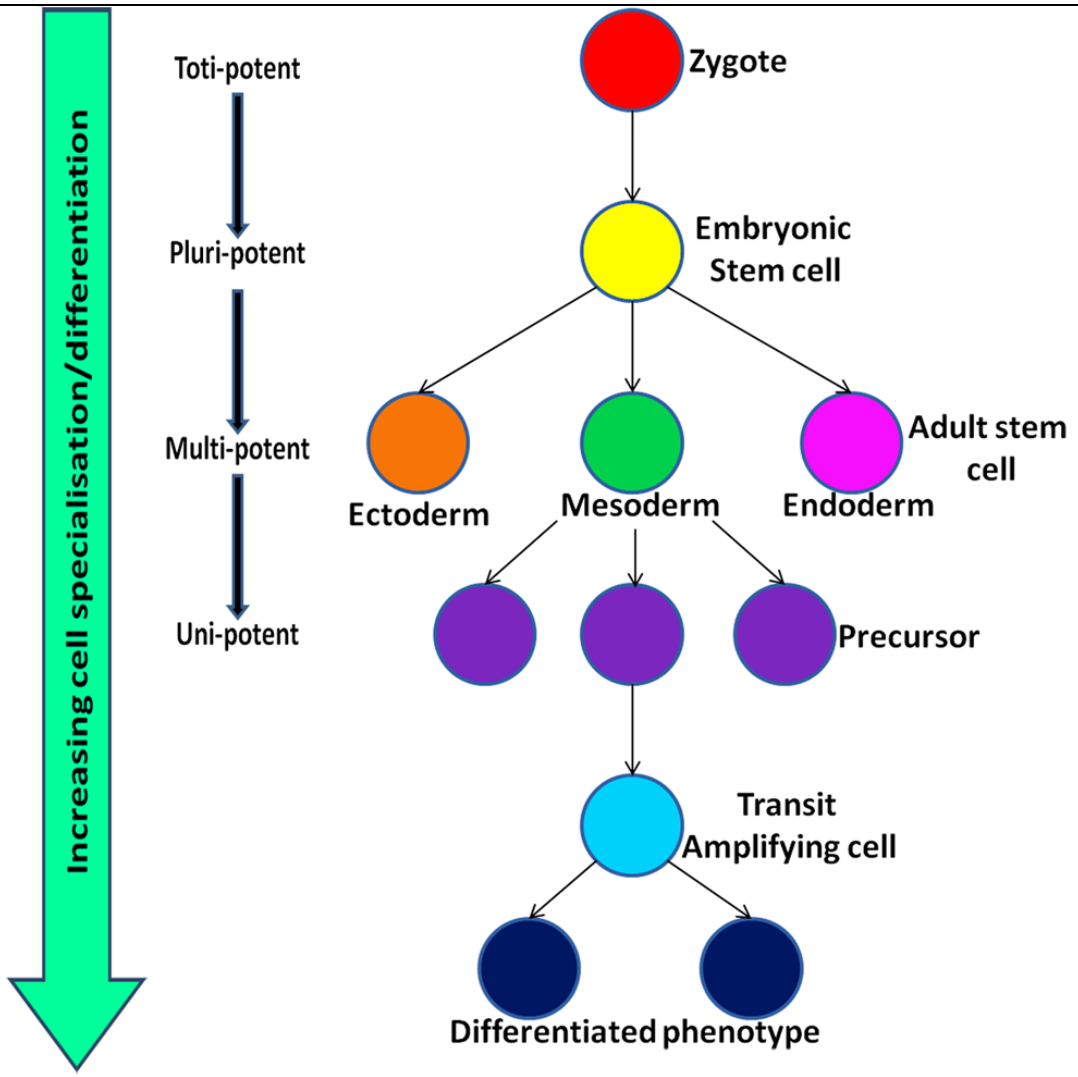
The first scientific research evidence on the existence of ASCs was produced more than 60 years ago. In the 1950s, researchers discovered that bone marrow contains at least two kinds of stem cells; haematopoietic stem cells, which form all the varieties of blood cells in the body, and bone marrow stromal stem cells (also called mesenchymal stem cells (MSC)), which were discovered a few years later. These non-hematopoietic stem cells make up a small proportion of the stromal cell population in the bone marrow, and can generate bone, cartilage, and fat cells that support the formation of blood and fibrous connective tissue.

The function of a stem cell in the literature seems to be dependent on the tissue, the context, and the groups of scientists who study the cells, thus, there is no general consensus amongst scientists as to the exact universally acceptable definition for them. Some researchers have proposed that a cell should have the following three qualities to qualify as a stem cell: clonogenicity (the ability of single cells to form colonies); prolonged self - renewal and proliferative potential (the ability to undergo a high number of passages before cell death); differentiation potential (the ability of a stem cell to produce differentiated progeny) (Eckfeldt, Mendenhall et al. 2005). For

simplicity, the different types of stem cells with reference to their differentiation potential are described below.

1.4.2 Types of stem cells according to their differentiation potential

The known hierarchical differentiation potential in stem cells is seen in Figure 1.9. The most potent of all mammalian stem cells is the zygote, which has the ability to differentiate into embryonic and extra-embryonic tissue and, therefore, is termed ‘totipotent’. *Totipotent cells* of the early embryo can build a complete organism, as well as extra-embryonal tissues such as the placenta (Nemeth and Karpati 2014).



Ectoderm	Mesoderm	Endoderm
Neurons	Connective tissues	Pulmonary alveoli
Glial cells	Skeletal muscle	Thyroid gland
Odontoblasts	Smooth muscle	Pancreatic cells
Epidermis	Urogenital system	Intestinal epithelium
Retina/lens	Adipose tissue	
Pigment cells	Blood cells	

Figure 1.9 - The graphical representation of the known hierarchical differentiation potential in stem cells, with a table depicting the progeny of all three germ layers.

Totipotent cells differentiate to produce pluripotent stem cells, which have the ability to form tissue from all three germ layers: the endoderm, mesoderm and ectoderm. Human Pluripotent stem cells are found within the inner cell mass (ICM) of the blastocyst. They are commonly referred to as embryonic stem cells (ESCs), and are capable of creating all tissues in the body (Nemeth and Karpati 2014). Human ESCs were first grown in the laboratory in 1998. ESCs provide enormous possibilities for disease research, and for providing new therapies. Unfortunately, transplantation of ESCs *in vivo* has led to teratoma formation, and acquisition of ESCs is associated with obvious ethical problems.

Pluripotent stem cells produce multipotent stem cells that give rise only to cell types within a particular germ layer; an example of this would be MSCs. Induced pluripotent stem cells (iPSCs) are obtained by reprogramming animal and human differentiated cells (Medvedev, Shevchenko et al. 2010). They are self-renewable and can differentiate to different types of adult cells (Singh, Kumar et al. 2015). The iPSCs are reprogrammed from somatic cells through ectopic expression of various transcription factors (Oct4, Sox2, Klf4 and c-Myc) (Singh, Kumar et al. 2015), and have provided the regenerative medicine field with a new tool for cell replacement strategies. The advantages over other sources of stem cells include, the generation of patient-derived cells, and the lack of embryonic tissue, whilst maintaining a versatile differentiation potential. Concerns regarding the delivery and expression of the reprogramming factors, the genomic instability, epigenetic memory and impact of cell propagation in culture, are ongoing (de Lazaro, Yilmazer et al. 2014).

Multipotent and unipotent stem cells exist in the embryo as well as in postnatal adult tissues. Multipotent stem cells can be found in the hematopoietic and in the stromal compartment of the bone marrow with the former producing blood lineages, and the latter being responsible for creating connective tissue cell types, such as adipocytes and osteoblasts (Nemeth and Karpati 2014). Unipotent stem cells can differentiate along only one lineage and are found in epithelial tissues, replenishing the epithelial cell

compartment of the skin, gut, or mucosal layer of the airways (Nemeth and Karpati 2014).

ASCs, synonymous with somatic stem cells (SSCs), are quiescent undifferentiated cells, existing in small numbers in most organs after the completion of embryonic development. They retain the potential to regenerate the entire tissue in which they reside. They differ from ESCs in that they are not pluripotent, they lack the ability to produce all cell types present in the embryo, and express different markers. This is obviously advantageous, since retention of multipotential undifferentiated cells in adult somatic organs that require regular regeneration, will increase the risk of teratoma formation. Although ASCs still possess high proliferative potential, and can persist for the individual's lifetime in many human tissues, they remain committed to a more specific lineage. ASCs are either unipotent, bipotent, or multipotent, and are able to reconstitute any part of a tissue by producing one or more of the component fully differentiated cells (Chan, Schwab et al. 2004).

ASCs undergo asymmetric cell divisions, which enable them to maintain their own population of undifferentiated cells (self-renewal), whilst also producing more differentiated, progenitor, daughter cells that are capable of rapid proliferation. They are fundamental units for tissue homeostasis, playing a critical part in the replenishment of dying cells, and regeneration of damaged tissue (Li and Xie 2005). The progenitors and their daughter cells proliferate, producing progressively more differentiated and mature progeny, characterised by the expression of more surface markers. These more mature cells, also known as transit-amplifying (TA) cells, are characterised by their limited proliferative potential and inability to self-renew, but are readily observed in tissue sections by expression of proliferation markers (Chan, Schwab et al. 2004). ASCs are a more attractive alternative to ESCs with regards to research and stem cell therapies as, comparatively, their acquisition is less controversial (without severe ethical concerns) and *in vivo* transplantation of them does not pose the risk of teratoma formation.

1.4.3 The stem cell niche

It is widely believed that adult tissue-specific stem cells reside within a “specialised microenvironment” known as the `niche`, and stem cell phenotype/ behaviour is regulated and maintained there (Spradling, Drummond-Barbosa et al. 2001). The niche senses the need for tissue replacement and compromises the ASC, surrounding niche cell(s), and extracellular matrix. It is a protective environment for the stem cell to maintain its genetic fidelity (Gargett and Masuda 2010). Furthermore, its function includes the balance of stem cell replacement (self-renewal), and provision of differentiated cells that are required for organ function (Gargett and Masuda 2010). The stem cells receive a unique combination of extracellular signals directing them to maintain their “stemness”, and preserving their stem cell characteristics (Fuchs and Horsley 2011, Nemeth and Karpati 2014). The non-stem cell residents of the niche must also be responsive to the needs of the organism/tissue so that they can integrate external cues and mobilise the stem cells to exit the niche during homeostatic replacement (tissue maintenance), or on injury (Fuchs and Horsley 2011).

A characteristic niche of epidermal stem cells is the bulge region of the hair follicle, they can also be found in the interfollicular epidermis. Hematopoietic stem cells are located either, in close proximity to osteoblasts, in the endosteal niche, or around sinusoidal vessels in the vascular niche within the bone marrow (Morrison and Scadden 2014). MSCs reside within the basement membrane of capillaries, and postcapillary venules, and form a subpopulation of contractile perivascular cells called pericytes (Sacchetti, Funari et al. 2007).

1.4.4 Methods of stem cell identification

Stem cell researchers often use one or more of the following methods to identify / characterise ASCs:

(1) Label the cells in a living tissue with molecular markers, and then determine the specialised cell types they generate.

(2) Remove the cells from a living animal, label them in *in vitro* cell culture, and transplant them back into another animal to determine whether the cells replace (or "repopulate") their tissue of origin.

(3) Use potential stem cell surface markers to isolate cells expressing the specific marker, and examine their functional stem cell characteristics *in vitro* and *in vivo*.

(4) Examine cells *in vitro* for their functional stem cell characteristics (e.g. clonogenicity, SP cells), and subsequently identify markers demarcating them.

The experimental methods follow the principle that it must be demonstrated that an ASC can generate a line of genetically identical cells that can give rise to all of the appropriate differentiated cell types of the tissue of origin.

In vitro functional assays used in the identification of putative stem cells are described below:

1.4.4.1 Colony-forming efficiency and holoclone identification

The colony-forming efficiency assay is an *in vitro* method that enriches for stem cells. Freshly harvested tissues are, initially, mechanically and enzymatically digested to release live cells, to produce a single cell suspension from the target tissue, followed by a dilution and inoculation of two dimensional (2D) culture dishes with a low number of cells (typically 10–100 cells/cm^2). The colonies that form are enriched in stem cells, and the number of resulting colonies is presumed to correlate with the initial number of stem cells in the single cell suspension, therefore, the initial tissue samples. When epidermal stem cells are studied, colonies formed by keratinocytes can be further sub cultured, and colonies in this secondary culture re-evaluated. Cells with high replicative capacity, and a low level of terminal differentiation, will form large colonies with smooth edges called holoclones. They represent a further enriched population of epidermal stem cells (Barrandon and Green 1987). Prolonged self-renewal requires serial passaging of cells before cell death and apoptosis. *In vitro* differentiation is performed by culturing cells in media, supplemented with various growth

factors, implicated at inducing differentiation into a specific cell type. Histochemical staining, along with gene expression, confirms differentiation.

There are other authors who are opposed to the use of clonogenicity to assign “stemness” to a cell population. They argue that stem cells have been assumed to spawn large, and/or diverse colonies, following explantation into tissue culture. The ‘logic underlying this expectation is flawed’, and ‘in most cases, “stemness” does not appear to be an autonomous cellular property that persists in culture, but a labile state maintained by the tissue microenvironment’. Without a physiological microenvironment, colony growth in culture may bear no relationship to cellular behaviour *in vivo* (Spradling 2011).

1.4.4.2 Side population

Side population (SP) analysis distinguishes stem and progenitor cells from other somatic cells, based on their ability to remove foreign molecules from the cells. The SP phenotype could be classified as either *in vitro* or *in vivo,* and was first described when studying bone marrow (Goodell, Rosenzweig et al. 1997). In a mixture of cells, DNA-binding fluorescent dyes (such as Hoechst 33342, a fluorescent dye that binds to the minor groove of deoxyribonucleic acid) are taken up by cells universally, but only stem/undifferentiated cells (expressing special ATP-binding cassette–containing pumps (ABC transporters - a type of membrane-bound active transporter)) will be able to remove the dyes. When excited by ultraviolet (UV) light, these fluorescence emitting dyes will remain unstained, or weakly stained, and therefore can be analysed, or isolated, by flow cytometry. Due to this mechanism, it is postulated that stem cells are able to extrude DNA damaging agents from their nucleus, where DNA resides. If stem cells had DNA damage, this would mean all progeny would be affected and it is, therefore, believed that stem cells have the ability to extrude Hoescht dye as a safety mechanism. In several tissues, dye-excluding SP cells represent a stem cell–enriched fraction. This method is able to pick up rare events, in a heterogeneous cell population. There are limitations with the SP assay: the detection of dye-excluding stem cells requires active cellular metabolism,

and is largely dependent on cell viability, which is adversely affected by the presence of DNA intercalating dyes; Hoescht can be toxic and the effect on cell survival can be poor, therefore, cells are counter stained with propodium iodide (PI) to ensure viability; there are many other dyes that are extruded using the same nuclear membrane pump, with reduced cellular toxicity, that have since been developed to aid more efficient isolation of these primitive populations (Bertoncello and Williams 2004, Telford, Bradford et al. 2007, Gisina, Lupatov et al. 2014, Tomiyasu, Miyamoto et al. 2014), despite this, Hoescht dye continues to be used; the isolated SP cells are not always suitable for further functional stem cell analysis (Terunuma, Jackson et al. 2003); fluorescence activated cell sorting (FACS) isolation requires isolation of cells in a single cell suspension, and this process is particularly detrimental to epithelial cells from a solid organ; it removes a stem cell from its niche, a process which may alter the retention of stem cell function, but force differentiation, therefore, the subsequent cell function examined *in vitro* of these FACS isolated cells may be different to their functional potential *in vivo,* or in the stem cell niche; there is growing caution that dye efflux ability is not ubiquitous, nor a common property, of all stem cell populations (Terunuma, Jackson et al. 2003, Triel, Vestergaard et al. 2004, Zeng, Park et al. 2009), the SP phenomenon has also been described in certain differentiated cells in adult tissues, so is therefore not restricted to the stem cell phenotype (Golebiewska, Brons et al. 2011); ABC transporters are expressed by specialised cells in several organs, including the small intestine (Mayer, Wagenaar et al. 1996), placenta (Lankas, Wise et al. 1998) and kidney (Smit, Schinkel et al. 1998), the transporters are thought to play a role in protection against the cytotoxic effects of toxins and xenobiotics by limiting toxin/drug entry into certain tissues, and promoting their elimination into the bile and urine (Fromm 2000).

Another FACS-based assay measuring mitochondrial membrane potential was shown to enrich cells with stem cell characteristics. By adding a potentiometric dye to a mixture of cells, followed by FACS analysis, stem cells can be separated by their higher mitochondrial membrane potential,

compared to more differentiated cells (Schieke, Ma et al. 2008, Charruyer, Strachan et al. 2012).

1.4.5 Isolation of cells using a variety of putative "stem cell" markers

1.4.5.1 Cell surface markers

Universal stem cell markers, if existed, would be very useful for the scientist studying stem cells and their properties, but unfortunately, to date, no such marker has been identified. Definitive markers of organ specific ASCs would facilitate identification and enrichment of cell populations of interest for further study. Cell surface markers have been utilised widely in a number of different tissues, but reports suggest different tissues to have many varied markers, including panels of markers. For example, the human hair follicle–derived epidermal stem cells were reported to be *negative* for CD45 and c-kit, *positive* for CD34 and CD200, and have a *low expression* of CD71, while producing α-6-integrin at a *high level* (Terunuma, Cross et al. 2008). The most widely reported markers of an undifferentiated/pluripotent state are NANOG, OCT4, and SOX2, but these are transcription factors, and are located within the cell nucleus. Cell isolation following their identification through a marker, then employing methods such as magnetic cell sorting (MACS), or FACS, can be used for functional studies of live cells, but in order to do this the marker is required to be on the cell surface, not in the nucleus. Permeation of the cell, needed to identify an intracellular marker, cannot be done on live cells, thus, no functional studies can be subsequently done in them.

1.4.5.1.1 Leucine-rich repeat-containing G-protein-coupled receptor 5

Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is a transmembrane receptor (Barker, van Es et al. 2007) characterised by a large leucine-rich extracellular domain (Barker, van Es et al. 2007), and belonging to a family of glycoprotein hormone receptors (Sun, Jackson et al. 2009). Little was known about mammalian LGR5 before 2007 (Barker and

Clevers 2010) when it was discovered by researchers looking to find an intestinal stem cell marker (Kumar, Burgess et al. 2014). R-spondins 1-4, a group of Wnt agonists, are the ligands of LGR5 (Carmon, Gong et al. 2011, Glinka, Dolde et al. 2011). LGR5 is closely related to LGR4 and LGR6 with approximately 50% sequence analogy. In comparison, it has 33% analogy to glycoprotein hormone receptors such as follicle stimulating hormone (FSH), LH, and thyroid stimulating hormone (TSH). LGR5 and LGR4 have 17 leucine-rich repeats in contrast to 13 in LGR6, and 9 in glycoprotein hormone receptors. The C-terminal flanking segment of LGR4 and LGR5 contains a cysteine-rich, chemokine-like domain, similar to the consensus CF3 subtype domain found in 45 glycoprotein hormone receptors. The core sequences of this consensus CF3 domain (CCAF and FK/NPCE sequences) are completely conserved, but the number of residues separating the conserved cysteines in LGR4 and LGR5 (CC-4X-C-4/54X-C) differs from that in the three known human glycoprotein hormone receptors (CC-15/23X-C-31/88X-C) (Kumar, Burgess et al. 2014). Similarly to FSH, the LGR5 ectodomain adopts a horseshoe-shaped architecture with C- and N-terminal caps (Figure 1.10). The linker between LGR5 repeats 10 and 11 has two phenylalanines at positions usually occupied by leucines. The binding site of R-Spondin 1 (RSPO1) on LGR5 is similar to the FSH binding site on the N-terminal leucine-rich repeat region of FSH, despite the ligands being quite distinct (Kumar, Burgess et al. 2014).

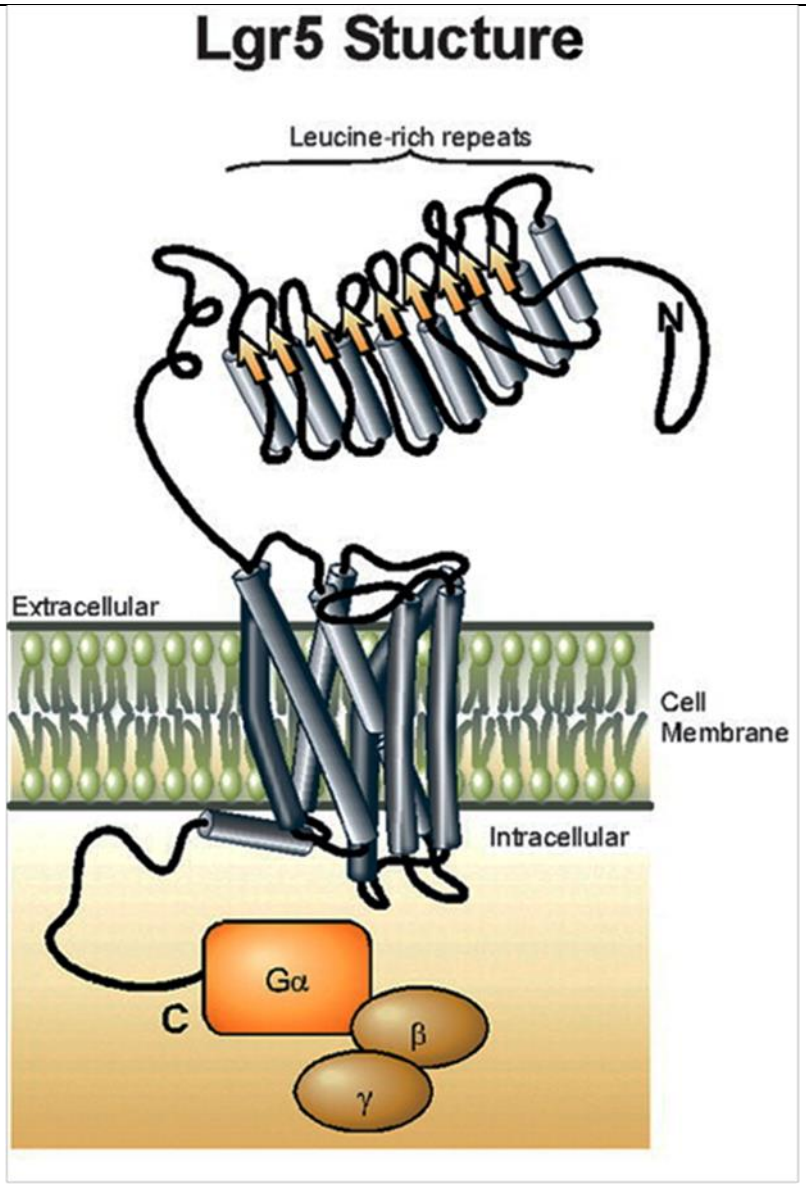


Figure 1.10 – The structure of LGR5.

Much of the understanding of LGR5 function has come from the analysis of null or loss-of-function mutants (Kumar, Burgess et al. 2014). Homozygous mice, lacking *Lgr5*, exhibit ankyloglossia and gastrointestinal tract dilation resulting in neonatal lethality (Sun, Jackson et al. 2009, Barker and Clevers 2010, Gil-Sanchis, Cervello et al. 2013). *Lgr5* was found to be expressed in the epithelium of the tongue, and in the mandible of wild-type embryos. The observed phenotype indicated that *Lgr5* is an essential gene, yet the lethal neonatal phenotype precluded the study of the role of *Lgr5* in adult tissues. The same *Lgr5*-null strain also had accelerated maturation of paneth cells in

the developing intestine (Garcia, Ghiani et al. 2009). The Wnt pathway is one of the pivotal molecular pathways in development, and is particularly involved in stem cell function. A role for *LGR5* in negatively regulating Wnt signalling during neonatal development of the intestine has been proposed (Schuijers and Clevers 2012, Kumar, Burgess et al. 2014). *LGR5* is considered a Wnt target gene (Sun, Jackson et al. 2009, Gil-Sanchis, Cervello et al. 2013), playing a key role in the regulation of the canonical Wnt signalling pathway and, therefore, in the activation/inhibition of cells occupying the niche (Gil-Sanchis, Cervello et al. 2013). RSPO protein expression, via *LGR5* receptor interaction, leads to enhanced Wnt activation (de Lau, Barker et al. 2011, Gil-Sanchis, Cervello et al. 2013). Understanding the critical molecular mechanisms associated with the RSPO:*LGR5* regulation of Wnt signalling is a key goal in stem cell biology. It is also important to determine whether the RSPO-*LGR5* complex activates intracellular signalling pathways independently of the Wnt-frizzled complex (Kumar, Burgess et al. 2014).

The role that the Wnt signalling plays in the physiology of the intestine has been identified for a long time, and it is suggested that one or more Wnt target genes could be stem cell markers (Barker, van Es et al. 2007). In 2007, Clevers' laboratory performed breakthrough research focused on the identification of the genes responsible for self-renewal in the small intestinal mucosa. From 80 genes identified as regulated by Wnt signalling, eleven demonstrated restricted expression to the intestinal crypt bottoms (Van der Flier, Sabates-Bellver et al. 2007). *LGR5* was the only gene expressed in the crypt in a distinctive manner. *LGR5* expression, demonstrated by *in situ* hybridisation (ISH), was restricted to a population of 10-14 proliferating slender cells, interspersed between the differentiated Paneth cells at the crypt base in the human small intestine, and on scattered cells at the base of the colonic crypts (Barker, van Es et al. 2007, Schuijers and Clevers 2012). These cells, known as crypt base columnar (CBC) cells, were discovered back in the early 1970s using detailed SEM studies (Cheng and Leblond 1974, Cheng and Leblond 1974). Largely on the basis of morphological considerations, Bjerknes and Cheng originally formulated the ‘stem cell zone’ model, proposing the CBC cells to represent the adult intestinal stem cell

population (Bjerknes and Cheng 1981). Formal proof of this model awaited the discovery of a specific marker for the cell population (Leushacke and Barker 2012).

To evaluate the "stemness" of *Lgr5*$^+$ populations, *in vivo* lineage tracing was used, and a heritable-inducible lacZ reporter gene was introduced into *Lgr5*-expressing cells. This, firstly, resulted in the appearance of lacZ$^+$ cells in the CBC compartment within the crypt base, over the course of a week the progressively expanding lacZ$^+$ progeny were observed extending from the crypt base towards the tips of the interstitial villi. Similar observations were also made in the colon. Thus, individual lacZ$^+$ tracing units were present in all epithelial cell lineages, and persisted throughout the life of the organism, identifying *Lgr5*$^+$ cells as a truly multipotent, self-renewing population of adult intestinal stem cells. Morphological and marker expression studies revealed that all differentiated cell types in the intestinal epithelium originated from *Lgr5*$^+$ CBC cells, therefore, that includes, enterocytes, paneth cells, goblet cells, enteroendocrine cells, tuft cells, and M-cells (Gerbe, van Es et al. 2011). Lineage tracing demonstrated *Lgr5*$^+$ CBC cells persisted over the lifetime of the animal, thus demonstrating that *Lgr5*$^+$ stem cells are long-lived and multipotent (Schuijers and Clevers 2012).

In vitro, small numbers of LGR5$^+$ cells are able to generate self-organising, self-renewing epithelial organoids with an architecture and cell composition that are remarkably similar to *in vivo* crypt/villus units (Sato, Vries et al. 2009, Leushacke and Barker 2012, Schuijers and Clevers 2012, Kumar, Burgess et al. 2014). Maintenance of these intestinal organoids is dependent on the presence of defined secreted growth factors, including RSPO1, noggin, and Epidermal growth factor (EGF) (Sato, Vries et al. 2009, Leushacke and Barker 2012).

It appears likely that generally, *LGR5*$^+$ stem cells double daily in tissue, and that adoption of stem cell or progenitor fate is determined stochastically (Kumar, Burgess et al. 2014). Intestinal stem cells divide symmetrically, such that every stem cell division gives rise to two equipotent daughters. These daughters can remain as stem cells or can become TA cells, depending on

their location relative to the Paneth cell niche. This model of ‘neutral competition' (that is, stem cells compete, but have equal chances to win) implies that longevity is an attribute of the stem cell population, but not of individual stem cells. The stem cell population size results directly from the size of the niche. Thus, the exact control of Paneth cell numbers at crypt bottoms is the central driver of homeostatic self-renewal (Schuijers and Clevers 2012).

The intestinal epithelium survives conditional ablation of the *LGR5*$^{+}$ stem cell compartment *in vivo*, but a dramatic reduction in *LGR5*$^{+}$ stem cell numbers accompanied the ablation of the Paneth cell compartment (Sato, van Es et al. 2011, Leushacke and Barker 2012). This finding led to the speculation that a dedicated ‘reserve’ *LGR5*$^{-}$ stem cell population may also exist (Kim, Escudero et al. 2012, Barker, Tan et al. 2013). An alternative explanation was provided when it was shown that secretory progenitor cells expressing the *Dll1* marker gene, which encodes the Notch ligand Delta-like 1, re-acquire a multipotent stem cell identity following damage-induced *LGR5*$^{+}$ stem cell ablation (van Es, Sato et al. 2012, Barker, Tan et al. 2013). These seminal works on *LGR5*$^{+}$ cells highlight many aspects of epithelial ASC biology as well as the possibility of the existence of more than one stem cell type in epithelial organs. The plasticity of the epithelial stem cell compartments, and their interaction with the surrounding niche, are under intense scrutiny in many human tissue systems at this present time.

1.4.5.1.2 N-cadherin

N-cadherin (CDH2 gene) is a calcium dependent cell adhesion molecule of the cadherin family; mainly mediating adhesion among the brain, muscle and vascular tissues (Xie, Zheng et al. 2017), and playing an important role in migration (Nguyen, Xiao et al. 2017). Expresssion of N-cadherin in epithelial cells is reported to be rare (Xie, Zheng et al. 2017), and induces changes in morphology to a fibroblastic phenotype rendering the cells more motile and invasive. N-cadherin can have multiple functions; promoting adhesion, or induction of migration, dependent on the cellular context (Derycke and Bracke 2004).

1.4.5.1.3 SSEA-1

In 1978, Solter and Knowles produced a monoclonal antibody from lymphocytes of a mouse immunised with F9 ECs, the monoclonal antibody, MC480, which recognised an antigen expressed by mouse ECs, and by the ICM of early embryos, and was down regulated on differentiation (Solter and Knowles 1979). The antigen, SSEA-1 was subsequently found to be the epitope recognised by the antibody Lewis-X hapten, a carbohydrate structure known to be related to the Lewis blood group antigens (Wright and Andrews 2009). In humans, the Lewis-X structure (also known as CD15) (Xu, Hardin et al. 2016) is expressed by some myeloid cells, and a variety of tumours unrelated to teratocarcinomas (Valentijn, Palial et al. 2013). Sialylated Lewis-X was found to be the ligand for E-selectin, one of a family of adhesion molecules involved in lymphocyte and macrophage homing to sites of inflammation and injury (Wright and Andrews 2009). Its expression on human ESCs is associated with differentiation (Valentijn, Palial et al. 2013). Lewis-X detecting antibodies are routinely used for cell sorting of neural stem- and progenitor cells. Applications include the enrichment of neural stem- and progenitors cells after neural differentiation of human iPSCs or ESCs, as well as their direct isolation from mouse neural tissue. Still, only a small amount is known about the role of Lewis-X in the central nervous system (Hennen and Faissner 2012). SSEA-1 may be expressed in normal ASCs and cancer stem-like cells (Xu, Hardin et al. 2016).

1.4.5.1.4 Musashi-1

The Musashi family is a conserved group of RNA-binding proteins (Potten, Booth et al. 2003), which regulates translation of target mRNAs specifically (Gotte, Wolf et al. 2008, Horisawa, Imai et al. 2010, Nikpour, Mowla et al. 2013). Musashi-1 promotes Notch signalling by binding to the mRNA of Numb, the negative regulator of Notch signalling, thus preventing its translation (Lagadec, Vlashi et al. 2014). The Musashi protein was originally identified as a regulator of asymmetrical division of sensory organ precursor cells in Drosophila (Nakamura, Okano et al. 1994, Nishimura, Wakabayashi et al. 2003, Potten, Booth et al. 2003).

Next, its mammalian homologs, Musashi1 (Msi1) and Musashi2 (Msi2), were identified in mice, prior to Musashi and Musashi-like proteins being discovered in a variety of multicellular animals, and the overall primary structure, and expression patterns, were highly conserved among them (Horisawa, Imai et al. 2010). Strong expression of Musashi1 protein has been identified in the nervous systems of vertebrates, in the central nervous system of mice and in undifferentiated neural stem/precursor cells at the embryonic and adult stages (Horisawa, Imai et al. 2010). Expression has also been observed in many types of somatic stem cells in adult tissues, e.g.: eye (Raji, Dansault et al. 2007); intestine (Potten, Booth et al. 2003); stomach (Akasaka, Saikawa et al. 2005); mammary gland (Clarke, Spence et al. 2005); and hair follicle (Sugiyama-Nakagiri, Akiyama et al. 2006). Mushashi-1 is, therefore, regarded as a marker for neural and intestinal epithelial stem cells (Murayama, Okamoto et al. 2009, Lan, Yu et al. 2010).

1.4.6 Tissue reconstitution

This methodology involves transplantation of donor cells into a recipient animal, to examine if the tissue of interest is reconstituted in the host. Most commonly, bone marrow derived stem cells are injected intra-venously, after labelling with (for example) enhanced green fluorescent protein, into immune compromised mice (Liu, Zhang et al. 2014). Alternatively, they can be transplanted subcutaneously, or under the kidney capsule. After a defined period of time, animals are sacrificed, and the explants can be examined (Masuda, Maruyama et al. 2007). These experiments, however, are limited by their absence of a stem cell niche, as the tissue of interest is transported without the surrounding tissue usually present in the natural location, potentially causing the explants not to develop into a functional organ.

Transplantation studies are important to define the differentiation potential of stem cells. These assays mimic a regenerative state that, in certain circumstances, forces stem cells to differentiate into lineages for which they usually do not contribute to regenerate to under physiological conditions (Van Keymeulen, Rocha et al. 2011).

1.4.7 Identifying stem cells for their in vivo properties

1.4.7.1 Label-retaining assays

In 1975, John Cairns proposed the immortal DNA strand hypothesis: when stem cells undergo mitosis, DNA is divided asymmetrically; the parent strand is retained by stem cells and the new strand is selectively segregated to a daughter cell. This mechanism would prevent accumulation of genetic errors, as it would mean that spontaneous mutations only occur in the newly synthesised strand, and are passed on to the stem cell progeny. This principle forms the basis for the label retaining cell technique (LRC). Parental DNA is marked with a DNA label during S phase of the cycle, typically bromodeoxyuridine (BrdU). If cells undergo symmetric mitotic division, the DNA label would rapidly be chased out of the cell, but if the cell undergoes asymmetric DNA division, the BrdU will segregate in the stem cell and would be detectable after a number of divisions. BrdU specific antibodies are then utilised to identify the LRCs in the tissue. The main drawback of LRCs is that BrdU binds to DNA during replication, instead of the usual thymidine, this means that mutations invariably occur. Additionally, BrdU is a recognised health hazard, therefore, the use of the LRC technique in humans is, justifiably, not permitted. Furthermore, more quiescent and non-dividing stem cells will not be labelled by this method. A pulse chase method is used to determine LRC characteristics, and enrich for stem cells. Firstly, in the “pulse phase”, radiolabelled nucleotide, or BrdU, is administered to animals, or cell cultures (for 3-5 days), labelling all proliferating cells in the S phase of the cell cycle (when DNA replication takes place). Following this, a long-term chase of the label (2–10 weeks) is undertaken to allow the highly proliferative cells to dilute the label, and the labelled cells that had terminally differentiated to be sloughed from the tissue. In this scenario, the amount of original label decreases to the point of being practically undetectable. LRCs are identified as they retain more label; they are slow-cycling and divide less frequently. Although this technique allows the *in situ* detection of a stem cell enriched population, it cannot be used to isolate living cells from tissue. The technique has been successful in its mission to identify cells that cycle

infrequently in the skin and the immune system (Punzel and Ho 2001, Potten 2004, Fuchs 2009), the hair follicle bulge (Cotsarelis, Sun et al. 1990) and the limbus of the eye (Cotsarelis, Cheng et al. 1989). The method relies on the stem cell population having at least some proliferative activity, entering the cell cycle during the period where they are labelled, and then subsequently being permeabilised to measure the level of nucleotide retention by FACS, rendering the cells non-viable for further assessments. Years later these hurdles are being defeated with modifications to the method, for example, the usual pulse chase was replaced by using the expression of tissue-specific, tetracycline-inducible histone, tagged with green fluorescent protein (H2B–GFP) (Tumbar, Guasch et al. 2004). Although this would allow isolation of the live green fluorescent protein+ LRCs cells for further functional analysis, the technique will still remain unsuitable for human *in vivo* studies.

1.4.7.2 Methylation patterns

CpG sequences are unmethylated at birth and become progressively methylated, over the years, from random errors that occur during cell division (Ahuja, Li et al. 1998, Yatabe, Tavare et al. 2001, Bird 2002). Therefore, more errors, or methylations, should be observed in cells with greater numbers of divisions since birth, regardless of why they have divided. Consequently, the hypothesis postulates that the number of divisions since birth (mitotic age) may be inferred by counting somatic errors (Ro and Rannala 2001).

Random replication errors in methlyation patterns are used to reconstruct somatic cell lineages (Kim, Tavare et al. 2005). This approach has been used to predict the progeny of stem cells, and their location, in lots of different systems including blood (Chambers, Shaw et al. 2007), brain (Nelson, Kavalali et al. 2008), intestine (Kaaij, van de Wetering et al. 2013), and endometrium (Kim, Tavare et al. 2005). This approach relies on unverified assumptions that replication errors are stochastic, and average error rates are constant. Somatic epigenetic errors are inherited, and methylation measurably increases with age in certain CpG-rich sequences

(Ahuja, Mohan et al. 1997, Yatabe, Tavare et al. 2001). Methylation can modulate transcription and chromatin structure, but some changes may reflect random errors, especially at CpG sites of unexpressed genes. Such age-related errors can potentially encode binary (methylated vs. unmethylated) information strings in adjacent CpG sites, creating tags that can be read with bisulfite sequencing (Yatabe, Tavare et al. 2001).

1.4.7.3 Lineage tracing

Lineage studies aim to establish which cells, and how many of those cells, will give rise to a structure in the early embryo and, as development proceeds, which part of a structure is responsible for the development of a substructure. Similar interrogations have now extended to the origin of stem cells that permit the regeneration of an adult structure, as well as its initial formation (Buckingham and Meilhac 2011).

Lineage analysis is a technique that was originally developed to study early embryos, and it represents, by far, the most powerful and reliable tool for identifying stem cells, and also for deciphering other aspects of tissue behaviour (Fox DT 2009). Lineage tracing can be utilised in both animals and humans, but the techniques utilised are very different. In order to follow the descendants of a parent cell, the recombinase approach to permanent genetic labelling by specific activation of a conditional reporter, is widely used in mouse and fly, and is also now available in fish, in addition to being used for genetic cell tracing in Xenopus (Satoh, Sakamaki et al. 2005). The techniques used to trace lineage in animal models are invasive, with a detrimental effect on the organism, thus, not suitable for humans. Consequently, a different way of tracking cell lineages had to be developed for human *in vivo* experiments.

1.4.7.3.1 Lineage tracing in Animal models

1.4.7.3.1.1 Drosophilia

Lineage tracing in the Drosophilia can be achieved by activation of a heat-shock promoter (hsp) regulating Flp (Harrison and Perrimon 1993), due to a change in temperature, or by the use of temperature-sensitive versions of the

GAL80 repressor (McGuire, Le et al. 2003). These control the activity of promoters, and enable cellular descendants of a singly marked cell to be monitored. Cell toxicity can also be a problem in Drosophila, therefore, Flp recombinases are now the preferred tools, and improved variants of Flp and Cre, together with the identification of specific target site variants (FRT, lox), have increased the efficiency and scope of these tools (Turan, Galla et al. 2011).

This strategy has been used to identify stem cells within the Drosophila ovary and posterior midgut. Using this method, intestinal stem cells were found to reside at the basement membrane, and be multipotent, producing both enterocytes and enteroendocrine cells. Regulation and differentiation systems for the intestinal stem cells were elucidated, and consisted of Notch, Janus kinase/signal transducer and activator of transcription, EGF receptor/mitogen-activated protein kinase, Hippo, and wingless signalling pathways (Singh, Mishra et al. 2012). Although drosophila work aided progression of stem cell research in some human organs, there were difficulties in translating the strategy to more complex human organs such as the endometrium.

1.4.7.3.1.2 Mice

In mice, regulation by temperature changes is not possible, and inducible systems (which involve antibiotics, such as the Tet system (Gossen and Bujard 1992), or hormones) have been developed. The ER, with tamoxifen as a ligand, is widely used (Feil, Boyano et al. 1997). Tamoxifen administration by injection into the mother usually results in recombination within 6–24 hours in the mouse embryo (Hayashi and McMahon 2002).

Two challenges exist for mouse lineage tracing, firstly, in engineering mice to express Cre under the control of a promoter that is only active in the desired stem cells, and secondly, titrating down the activation of Cre within the stem cell niche such that on average, only one cell per niche is marked. The stem cell that is initially labelled will be long-lived and recognisable based on its mark and location, if it remains in its niche. When the engineered mice express an inducible Cre recombinase in the desired cell type, they are then

mated to a different mouse, genetically manipulated to ubiquitously express Rosa26 gene. The resulting offspring will have a stop codon (or secondary gene) flanked by Cre-recombinogenic loxP sites, followed by a reporter gene positioned downstream from the Rosa26 promoter. Once the Rosa26 locus has been recombined, reporter activity is faithfully transmitted to all progeny, and a small clone of marked cells will develop that will continue to increase in size over time. The progeny are expected to be shorter lived, leaving a trail of transiently labelled cells, enabling a tracing of where they go, and what lineages they differentiate into. This analysis was employed in the hair follicle, demonstrating that self-renewal and differentiation are separated temporally in follicular stem cells. The problems with this method are related to the toxicity of Cre (Naiche and Papaioannou 2007). Most promoters active in stem cells are not exclusive to these cells, resulting in a heterogeneous group of stem cells, and early progeny, that are marked; and long-term quiescent stem cells, or stem cells leaving the niche, that are not revealed with this method.

Clevers lab knocked an EGFP–ires–CreERT2 transgene into the *Lgr5* locus. Mice carrying this locus were then crossed to Rosa26–fl–stop–fl–lacZ reporter mice, and the resulting strains were treated with tamoxifen to activate Cre in only a small number of *Lgr5*-expressing stem cells at the base of the intestinal crypts, which resulted in excision of the floxed stop codon in the Rosa-lacZ reporter. GFP^+ and βgal^+ stem cells remained localised, but over time entire discrete crypts were found to be βgal^+, but did not express GFP, suggesting that they were progeny of the *Lgr5*-expressing cells (Barker, van Es et al. 2007).

In the hair follicle system, lineage tracing using Lrg5-Cre mice to label hair follicle stem cell progeny that have moved away from the bulge, showed that committed cells that have progressed along the lineage and lost stemness, are able to home back to their niche. When back in the niche, these non-stem cells transmit critical inhibitory signals to the stem cells that keep them in a quiescent state, even in the presence of positive stimuli, and raise the threshold of activators needed to initiate the next round of stem cell activation and hair production (Hsu, Pasolli et al. 2011). This demonstrates

the importance of instructing stem cells when to stop tissue regeneration, as well as initiating the regenerative process, during tissue homeostasis and wound repair. By imposing both stimulatory and inhibitory signals on the stem cell niche, the balance between quiescence and activation of its residents is placed under the dynamic control of the microenvironment.

1.4.7.3.1.3 Mosaics: Labeling of several progenitors simultaneously

Labelling of several progenitor cells may be useful to assess the variability of cell fate potential, to recognise differing cell fate choices after asymmetric division, or to monitor cell dynamics with genetic manipulations to produce mosaics classically used in Drosophila for clonal analysis. This would depict the existence of multiple stem cells in a tissue. From pioneering work by Sturtevant (1929) on gynandromorphs (an organism that contains both male and female characteristics) resulting from spontaneous X-inactivation, to current techniques for spatially and temporally controlled activation of cell markers, the coherent growth of cells in the Drosophila embryo has facilitated clonal analysis. Isolated clusters of labelled cells are generally assumed to be clonal.

The Brainbow system, developed for mouse models, depends on a stochastic choice between distinct recombinase target sites flanking a range of fluorescent markers in a transgene integrated in multiple copies. This technique leads to the generation of a spectacular mosaic of differently coloured cells (Livet 2007). With fluorescent proteins targeted to subcellular compartments, as well as with recombinase-mediated inversion of reporter sequences (Brainbow 2), the possible combinations that can distinguish cells are huge. A universal rainbow line, R26R-confetti (Snippert, van der Flier et al. 2010), coupled to existing specific Cre lines, increases the range of applications. This was used with a Cre-ERT2, under the control of *Lgr5*, which is expressed in stem cells of the crypt of the mouse intestine. Tamoxifen induction of Cre at different time points, followed by mathematical analysis of cell patterns marked with the four randomly generated reporters, led to conclusions about stem cell turnover without asymmetric cell divisions. Stochastic adoption of stem, or TA cell fates, depends on neutral competition

between cells. Such sophisticated mathematical analysis of clone distributions has also been applied to other tissues, and a general theoretical framework, which discriminates between patterns of long-term clonal evolution for distinguishing three classes of stem cell behaviour, has been proposed (Klein and Simons 2011). A limitation to this method is in the analysis, the sorting of colours can be limited by the spectral separation of different combinations of fluorescent proteins, and by the light microscopy resolution of subcellular localisation. Mosaics have, nonetheless, given insight into the polyclonal origin of tissues, their architecture, and the cell dynamics underlying tissue growth.

Following the *Lgr5* lineage tracing experiments in the small intestine, it was thought that *Lgr5* marks intestinal stem cells, and that an individual stem cell can give rise to an individual crypt unit. To test this hypothesis further, Clevers and colleagues employed Brainbow technology. After inducing Cre expression in *Lgr5*-expressing stem cells, the resulting mix of colours revealed that intestinal stem cells do indeed generate clonality in the small intestine (Snippert, van der Flier et al. 2010). Such experimental work highlights the importance of lineage tracing in identifying stem cells and their involvement in tissue regeneration.

1.4.7.3.2 *In vivo* lineage tracing in humans

1.4.7.3.2.1 Mitochondrial DNA mutations

The adaptation of lineage tracing to humans was reported first when Taylor and colleagues in Newcastle-upon-Tyne (Taylor, Barron et al. 2003) originally used the mitochondrial DNA (mtDNA) mutations method on human colon tissue. The method was later generalised for the identification of human epithelial stem cell niches in solid organs by Fellous et al (Fellous, McDonald et al. 2009) and we have, therefore, employed this well-established method to prove the existence of human endometrial epithelial stem cells for the first time. In this context, the analysis of passenger mutations has proven to be more useful than the analysis of driver mutations, because maintenance of passenger mutations is presumably not subject to selective pressure (Fearon and Bommer 2011). Tracing mtDNA mutations is

deemed a natural experiment, genetic changes occur spontaneously, allowing lineages to be tracked and, in several instances, the stem cell niche to be identified (Wright 2012). The following section explains this methodology in detail.

1.4.7.3.2.2 Mitochondria

Mitochondria are essential intracellular organelles, required for oxidative phosphorylation and found in all nucleated human cells. They contain the only non chromosomal DNA in human cells (their own mtDNA) (Taylor and Turnbull 2005), and are, therefore, under the dual control of nuclear DNA and mtDNA (Taylor, Barron et al. 2003). Each cell in the body can contain hundreds, or thousands, of mitochondria, and each mitochondrion contains multiple copies of its own mitochondrial genome (Wallace 2005, Fellous, McDonald et al. 2009). The mtDNA is a small (16.5 kb in humans), self-replicating DNA molecule, that encodes 13 essential proteins of the mitochondrial respiratory chain, as well as 2 ribosomal RNA and 22 transfer RNA genes required for intra-mitochondrial synthesis of proteins (Anderson, Bankier et al. 1981). All of the other mitochondrial proteins are nuclear encoded and transported into the mitochondria. Unlike nuclear DNA, mtDNA is not integrated with the cell cycle (Elson, Samuels et al. 2001).

The mitochondrial genome is more prone to mutations than nuclear DNA due to its high production levels of reactive oxygen species (ROS) (Richter, Park et al. 1988), and mitochondria lack the protective histones and DNA repair mechanisms that protect the nuclear genome (Taylor, Taylor et al. 2001, He, Chinnery et al. 2002, Fellous, McDonald et al. 2009).

1.4.7.3.2.3 Mitochondrial mutations

Inherited abnormalities of the mitochondrial genome are recognised as important causes of disease (He, Chinnery et al. 2002, Taylor and Turnbull 2005) and are common, with over 200 pathogenic defects (point mutations, deletions, and rearrangements) having been identified (Taylor, Taylor et al. 2001, He, Chinnery et al. 2002). mtDNA, being prone to mutations, also carry spontaneous non-pathogenic passenger mutations. Through these, visualisation of the progeny of an individual stem cell in a tissue section has

been realised, where these passenger mutations are used as clonal markers (Walther and Alison 2016). The passenger mutations are passed on to daughter cells during cell division, providing a platform for studying cell lineage in human tissues. Although these mutations are generally thought to have no functional consequence, there is recent evidence that they may contribute to stem cell aging, suggesting tissue aging is driven by stem cell dysfunction in response to the accumulation of mtDNA point mutations (Su, Turnbull et al. 2018).

The mitochondrially-encoded cytochrome C oxidase gene (CCO), which confers little or no selective growth advantage to normal cells (Nooteboom, Johnson et al. 2010), is a spontaneous passenger non-pathogenic mutation, which occurs in the enzyme forming the last step of the electron transport chain in respiratory complex IV (Mootha, Lepage et al. 2003, Taylor, Barron et al. 2003, Greaves, Preston et al. 2006, Li, Park et al. 2006, McDonald, Greaves et al. 2008). It is also a major regulatory site for oxidative phosphorylation, which is essential for the assembly and respiratory function of the enzyme complex (Li, Park et al. 2006).

When CCO mutations occur, they can affect all copies of the mitochondrial genome within a cell, termed homoplasmy (homoplasmic conversion, where all copies of the genome in a cell are identical, and a mutation in the mtDNA will be present in all copies); or there may be a mixture of mutated and wild-type genomes in the same cell, termed heteroplasmy (mixed mitochondrial genotype where a mutation is present in only some copies of the genome) (Lightowlers, Chinnery et al. 1997, Taylor and Turnbull 2005). In the presence of heteroplasmy, due to the mitochondria containing multiple mtDNA copies, and the cells containing hundreds or thousands of mitochondria, at least 80% of the CCO genes in a cell must be mutated (Taylor, Barron et al. 2003) for the visualisation of a detectable deficiency in CCO enzyme activity. This will allow direct visualisation of cell lineages in a renewing tissue ASC system (Sciacco, Bonilla et al. 1994, Taylor, Barron et al. 2003).

1.4.7.3.2.4 Expansion of mtDNA mutations

The expansion of a mtDNA mutation within a cell can follow a stochastic course, which, over time, can evolve from a heteroplasmic state to a homoplasmic, or near homoplasmic, state. This process is termed “genetic drift” (Elson, Samuels et al. 2001, Wright 2012). The mutation initially spreads within the individual mitochondrial genome, and then in the mitochondria of the stem cell, to a level of homoplasmy (80% of CCO genes must be mutated for a cell to show as negative). Therefore, due to this lengthy process, wholly-mutant crypts are rarely seen before the age of 40 years (Greaves, Preston et al. 2006), an indication of the time required (Greaves, Preston et al. 2006, Wright 2012). Stem cells should be the oldest cells in an organ. Due to genetic drift taking many years, we assume that only stem cells are sufficiently long lived in a tissue, to acquire the near-homoplasmic state, enabling detection of a biochemical deficiency in them (Fellous, McDonald et al. 2009).

1.4.7.3.2.5 Using CCO to demonstrate the potency of human stem cells

In tissues such as the small intestinal crypt and the gastric gland, CCO^- crypts and glands were shown to contain cells of all lineages in the epithelial system, showing that all the cells in these glands originated from a parent CCO^- stem cell (Wright 2012).

This method has been utilised to confirm the existence of epithelial stem cells in the colon (Taylor, Barron et al. 2003), pancreas (Fellous, McDonald et al. 2009), bladder (Gaisa, Graham et al. 2011) and prostate (Gaisa, Graham et al. 2011), but human endometrium has not yet been examined with this method.

1.5 Evidence for endometrial stem cells

The human endometrium is a highly regenerative organ undergoing over 400 cycles of shedding and regeneration over a woman's life time (Jabbour, Kelly et al. 2006, Gargett 2007, Cervello, Gil-Sanchis et al. 2010). Menstrual shedding, and subsequent repair of the endometrial functionalis, is a process unique to humans, and higher-order primates (Slayden and Brenner 2006, Brenner and Slayden 2012). It re-grows from 1-2 mm after menstrual shedding to 16 mm in the secretory phase of the menstrual cycle (Fleischer 1999), and is able to completely regenerate after parturition, and in post-menopausal (PM) women, when exposed to oestrogen replacement therapy (Gargett 2006, Gargett 2007). Even after extensive iatrogenic destructive procedures such as ablation (Tresserra, Grases et al. 1999), the endometrium regrows in some women, who will continue to bleed (25-75%) (Gimpelson 2014, Muller I 2015). This huge regenerative ability suggests that the endometrium has a stem cell(s) basis that supports the tissue maintenance/regrowth.

1.5.1 History of endometrial stem cells

Prianishnikov was the first to consider the existence of endometrial ASCs and, in 1978, he proposed ASCs to reside in the deep basalis layer, and their differentiation to be marked by functional changes (acquiring) in hormonal receptivity (Prianishnikov 1978). He suggested a hierarchical hormone receptiveness in the endometrial cells matching their level of maturity, therefore, the most primitive, hormone independent ASCs differentiate initially into oestrogen dependent cells, then they may further differentiate in to both oestrogen and progesterone dependent cells. Terminally-differentiated cells were expected to be only progesterone dependent, and postulated to have a limited lifespan (Prianishnikov 1978) (Figure 1.11).

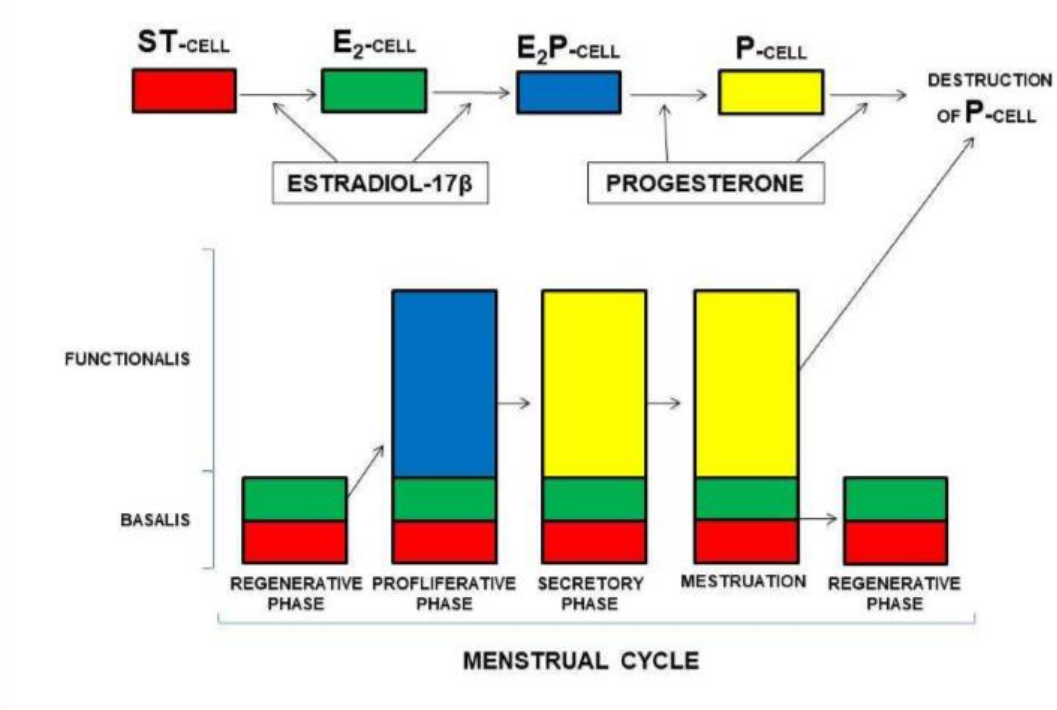


Figure 1.11 – Hormone dependent regulation of the ratio of cell types in the endometrium (Abbreviations - ST-cell – stem cell, E2-cell – oestrogen sensitive cell, E2P-cell – oestrogen-progesterone sensitive cell, P-cell – progesterone sensitive cell). Figure adapted from (Prianishnikov 1978).

This hypothesis, proposing that the human endometrium regenerates from the deeper basalis layer (which is the proposed germinal compartment that persists after menstruation, and is responsible for the regeneration of the new upper/ superficial functionalis layer), has been echoed many times since (Chan, Schwab et al. 2004, Valentijn, Palial et al. 2013, Fayazi, Salehnia et al. 2016, Gargett, Schwab et al. 2016).

Identifying human endometrial epithelial stem cells is problematic, due to the lack of specific markers for isolating and examining them for functional properties (Kim, Tavare et al. 2005). Furthermore, the true, and conclusive, confirmation of an endometrial epithelial stem cell requires the demonstration that they are able to produce all of the epithelial cell types that exist in all regions of the endometrium. The characterisation of different endometrial epithelial cell subtypes to ascertain the mature progeny of the putative stem cell is not yet complete in the human endometrium.

1.5.2 Evidence so far for the existence of endometrial stem cells

1.5.2.1 *In vivo* evidence

1.5.2.1.1 Scanning Electron microscope studies

Early work on endometrial regeneration has been gleaned through SEM studies, this includes some evidence for the existence of endometrial epithelial stem cells. SEM studies confirmed that the total regeneration time for postmenstrual surface re-epithelialisation to be approximately 48 hours (Ferenczy 1976) (with the regenerative period beginning between cycle days 2 and 3, and ending between cycle days 4 and 5). This time interval coincides with the maximum area of the denuded basalis (Ferenczy 1976). The SEM data also suggests that the surface epithelial repair occurs by a simultaneous and progressive outgrowth from the remaining stumps ("mouths") of basal glands, and by out growth from the surface epithelium, adjacent to the denuded areas, that has not been lost from the isthmic and cornual regions (Ferenczy 1976). A similar observation was reported in rabbit endometrium; the newly restored surface lining was derived from the intact surface epithelium that remained bordering the artificially (rabbits do not menstruate) denuded area (Schenker, Sacks et al. 1971). The other important findings of the SEM studies had been the description of morphological differences in endometrial epithelial subtypes. For example; ciliated and non-ciliated cells have been observed in the LE (Baraggino, Dalla Pria et al. 1980). Cells with the same morphology as that of endocrine cells were found in the lower layers of the epithelium at late gestation (clear cells), and they were positive for anti-serotonin and anti-somatostatin antisera (Satake and Matsuyama 1987), and morphologically similar to endocrine cells in other tissues (Wang 1989). This further confirms the existence of different epithelial subtypes that are yet to be characterised for the expression of distinct markers or for their possible functional diversity.

1.5.2.1.2 Studies using stem cell markers

Stem cell marker identification in the endometrium has been undertaken according to 3 main hypothesis:

1. Examining endometrium for the expression of *putative stem cell markers* that were identified to be expressed in the ESCs, or stem cells of different tissues (e.g. *OCT4, Mushashi-1, LGR5*).

2. Identifying markers that are highly expressed in cells with some *in-vitro stem cell properties* (clonogenic cells, SP cells).

3. Identifying markers that are expressed by cells *located in the postulated stem cell niche* (basalis and PM glandular epithelium).

1.5.2.1.2.1 Putative stem cell markers

1.5.2.1.2.1.1 OCT-4

OCT-4, a marker of pluripotent human ESCs and some ASCs, was seen in some endometrial samples by IHC, and in all endometrial samples by reverse transcriptase- polymerase chain reaction (RT-PCR) with variable expression in the human endometrium (Matthai, Horvat et al. 2006). Close scrutiny of the pictures presented in this manuscript reveal that OCT-4 is not expressed in the epithelial cells, but appears to be seen rarely in some stromal cells or blood vessels. The presented pictures are not conclusive, and there is no further explicit description, or further confirmatory data using a different method in the manuscript, about the above findings. The authors simply concluded that OCT-4 staining is present, and mostly expressed in the stromal compartment (Matthai, Horvat et al. 2006). OCT-4 was found not to be differentially expressed during the menstrual cycle in women, therefore, proposed to be uninfluenced by hormones (Bentz, Kenning et al. 2010). OCT-4 has also been located in some mouse LRCs in the lower region of the stroma, co-localising with c-KIT (Cervello, Martinez-Conejero et al. 2007). Therefore, further work needs to be done using a reliable antibody, specific to human OCT4a antigen, to examine the cell specific expression in the human endometrium, since this is the particular antigen associated with an undifferentiated phenotype.

1.5.2.1.2.1.2 Musashi-1

Musashi-1, an RNA-binding protein in neural stem cells and an epithelial progenitor cell marker that regulates self-renewal signalling pathways, was immunolocalised to single epithelial cells, and small clusters of stromal cells in human endometrium (Gotte, Wolf et al. 2008). The authors describe the staining as nuclear and cytoplasmic, but the representative figures presented in the manuscript only demonstrated cytoplasmic staining. Immunofluorescence (IF) images showed Musashi-1 to be co-localised with its molecular target, Notch-1, and telomerase. Musashi-1 positive cells were mainly found, in the basalis, in the proliferative stage of the menstrual cycle (when compared to the secretory stage), suggesting their possible stem/progenitor cell function. Stromal Musashi-1 positive cells were not found in a perivascular location, although some were in a peri-glandular region, a similar location to some stromal LRC in mouse endometrium (Chan and Gargett 2006).

More recently, Musashi-1 expression has, been found in the neonatal endometrium from the twelfth week of gestation, with the number of positive cells decreasing with increasing gestational age. In reproductive endometrium Musashi-1 staining was seen in dispersed single cells, and in stromal cell groups adjacent to myometrium (Lu, Lin et al. 2011).

In summary, there is unconvincing cytoplasmic and nuclear staining for Musashi-1, without any functional studies, and no further confirmatory follow up work has been undertaken since the preliminary work published in 2006. Taken together, the above evidence suggests Musashi-1 has not proven itself as an endometrial stem cell marker.

1.5.2.1.2.1.3 SOX9

SOX9 is a Wnt target transcription factor first discovered in patients with campomelic dysplasia (Nguyen, Sprung et al. 2012). SOX9 expression differentiates cells derived from all three germ layers into a large variety of specialised tissues and organs (Jo, Denduluri et al. 2014), with roles in chondrogenesis (Foster, Dominguez-Steglich et al. 1994, Wagner, Wirth et al. 1994) , male gonad development (Sudbeck, Schmitz et al. 1996), neural

crest development (Spokony, Aoki et al. 2002) and in the lower crypt region of the intestinal epithelium (Blache, van de Wetering et al. 2004, Bieczynski, Torres et al. 2016). SOX9 expression detected by IHC in normal human endometrium was found to be significantly higher in the proliferative phase of the menstrual cycle when compared with the secretory phase (Saegusa, Hashimura et al. 2012). Following this work, another IHC study, described SOX9 expression to be largely confined to the basal epithelial cells throughout the cycle, with significantly greater numbers of epithelial cells expressing nuclear SOX9 in the basalis (46.2–52.3%) over the functionalis (8–12.1%) glands. The PM endometrium demonstrated the highest SOX9 immunostaining out of the pre/PM endometrial samples, with over 75% of PM epithelial cells expressing nuclear SOX9 (Valentijn, Palial et al. 2013).

This paper concluded that nuclear SOX9, co-localised with SSEA-1 and nuclear β-catenin suggesting an activated Wnt pathway, in the basal glands of premenopausal endometrium, could function by maintaining the SSEA-1 cells in a less-differentiated state, representing the endometrial stem/progenitor cell compartment of the stem cell niche, playing an important role in homing stem cells (Valentijn, Palial et al. 2013). It was also concluded that high levels of nuclear SOX9 observed in the PM endometrial epithelial cells may function as a checkpoint to prevent hyperplasia, as loss of SOX9 in the intestinal epithelium leads to hyperplasia (Bastide, Darido et al. 2007). Although nuclear SOX9 may mark a potential primitive cell population in the endometrium, due to the nuclear location, SOX9 is obviously not a suitable marker to select these cells for further functional studies. The authors further concluded that nuclear SOX9, containing basalis endometrial epithelial cells, can be isolated for functional studies using the surface marker SSEA-1 (Valentijn, Palial et al. 2013), and their subsequent in vitro experiments demonstrated these epithelial cells to have high telomerase activity and superior ability to generate endometrial gland-like organoids in 3D culture (Valentijn, Palial et al. 2013).

1.5.2.1.2.1.4 LGR5

By using RT-PCR, *LGR5* gene was found to be expressed in 26 full thickness, mid proliferative to late secretory, human endometrial samples. Substantial differences were discovered in expression levels of individual women, but no variation was observed throughout the menstrual cycle, therefore suggesting it was not hormonally regulated (Krusche, Kroll et al. 2007). This study was followed by a mouse study demonstrating that: *Lgr5* gene is dynamically regulated in endometrial epithelium; expressed only in immature and ovariectomised mice; and down-regulated by oestrogen. All of this evidence alludes to a hormonal regulation, and *Lgr5* to be lost with differentiation (such as acquiring hormonal responsiveness) (Sun, Jackson et al. 2009). More recently, in a review paper, a group from Valencia proposed LGR5 to be a potential stem cell marker in the human endometrium. They included original data, using IHC, to show a population of stromal and epithelial cells stained by an anti-human LGR5 antibody, mainly to perivascular regions (Gil-Sanchis, Cervello et al. 2013). In this review, data from telomapping (a type of confocal quantitative fluorescence *in situ* hybridisation that displays the gradient of telomere length in a given adult tissue) was also included, it was used to identify cells with the longest telomeres. The authors subsequently claimed that *LGR5* mRNA signal was present in some of the cells containing the longest telomeres, suggesting LGR5 expressing cells are associated with a long telomere phenotype. However, it must be noted that this statement was made without the data being presented in the paper. No menstrual cycle differences were noted with regards to expression of LGR5 protein. This work concluded that, perhaps, LGR5 could be considered as a universal stem cell marker (Gil-Sanchis, Cervello et al. 2013). This paper, being a review (and although mentioning some of their original work on *LGR5* mRNA and protein), does not present the data to a scientifically acceptable standard. That is, it does not provide the details of methodology or information with respect to the exact antibody used (Gil-Sanchis, Cervello et al. 2013). Since the specificity of the available anti-human LGR5 antibodies are in a considerable doubt, this is a significant deficiency in this manuscript, which casts a huge doubt on

the credibility of claims made by the authors. The same group later published another study, xenografting isolated cells expressing LGR5 into a mouse model (Cervello, Gil-Sanchis et al. 2017). An anti-human LGR5 antibody was used to isolate positive cells from human endometrium to determine their functional relevance, with no attempt at locating the positive cells in the intact human endometrium pre-sorting. It is unclear if the authors used the same antibody (or not) that they had used in the IHC data presented in the review article (Gil-Sanchis, Cervello et al. 2013). Human $LGR5^+$ epithelial and stromal cells from endometrial biopsies (not full thickness endometrium, thus we assume only containing functionalis including LE) were sorted, according to their surface expression of LGR5, using an antibody (of unconfirmed specificity) by FACs, and phenotypically characterised by flow cytometry with haematopoietic and mesenchymal markers. These cells were labelled and injected under the kidney capsule of immunocompromised mice. The authors reported $LGR5^+$ cells in the human endometrium to constitute 1.08 ± 0.73%, and 0.82 ± 0.76% of the total cells in the epithelial and stromal compartments respectively. $LGR5^+$ cells showed an abundant expression of CD45 (a hematopoietic marker), and no expression of surface markers CD31, CD34, CD133, CD73, and CD90. However, co-expression with the macrophage marker CD163 was detected. The tissue recapitulation resulted in a weak endometrial reconstitution, and transcriptomic profiling revealed new attributes for $LGR5^+$ cells, related to their putative hematopoietic origin. The authors concluded that LGR5 was unlikely to be a universal stem cell marker, contradicting their proposal made in the previous review paper (Gil-Sanchis, Cervello et al. 2013). They further stated that $LGR5^+$ cells appeared to be recruited from blood to be part of the stem cell niche at the perivascular microenvironment, to activate the endogenous niche (Cervello, Gil-Sanchis et al. 2017). The conclusions, and main criticisms drawn from the current literature on all previous endometrial LGR5 expression studies therefore are: Mouse studies may not translate well to humans due to obvious species-specific differences in their endometrial biology; the initial study examining *LGR5* mRNA level did not attempt to ascertain the location of the endometrial *LGR5* expression; the specificity of all presently available anti-

human LGR5 antibodies to identify the protein are not confirmed, and are of considerable doubt (Munoz, Stange et al. 2012).

Therefore, further work is necessary to ascertain the exact endometrial cell types that express *LGR5*, their location, phenotype and functional characterisation. Testing the utility of LGR5 as a marker of human endometrial epithelial progenitor cells will also require the development of high-quality antibodies to a surface epitope, to purify the cells for subsequent assessment of their ASC function.

1.5.2.1.2.2 Basalis specific markers

1.5.2.1.2.2.1 SSEA-1

SSEA-1 is a cell surface glycan; an antigenic epitope; defined as Lewis X carbohydrate; and is expressed by preimplantation mouse embryos, teratocarcinoma stem cells, and mouse ESCs (Knowles, Aden et al. 1978, Solter and Knowles 1978, Knowles, Rappaport et al. 1982, Fox, Damjanov et al. 1983). Its presence signifies cells in an undifferentiated state, as expression is lost during stem cell differentiation.

In the endometrium, immuno-reactivity to SSEA-1 was specific to epithelia only. Intensity was significantly greater in the proliferative over the secretory endometrial phase, and strongest in the basalis, and basalis like, PM endometrium, when compared to the functionalis epithelium. SSEA-1 enriched cell populations have a greater propensity to produce gland-like structures in 3D culture, and also have higher telomerase activity and longer telomere lengths. The function of SSEA-1 in the endometrium remains unknown, but it is postulated to be associated with cell adhesion, migration, and capacity to differentiate (Valentijn, Palial et al. 2013).

Fibroblast growth factor (FGF) and Wnt-1 are both involved in stem cell maintenance and differentiation, work has shown that SSEA-1 possibly functions to bind and modulate these growth factors (Capela and Temple 2002), and when this is supplemented with the prominent expression of SSEA-1 in the basalis epithelium, it is conceivable that these cells are a component of the endometrial-epithelial stem/progenitor cell niche.

1.5.2.1.2.2.2 N-cadherin

The first report of human endometrial expression of N-cadherin was in 2010, comparing the endometrium of infertility patients to fertile controls. N-cadherin staining was at the epithelial apical and lateral membrane, but was very weak (H-score <1) in the endometrium, conversely to this, N-cadherin staining was strong in the tubal epithelium. N-cadherin expression did not change with the menstrual cycle, and the authors concluded that N-cadherin could have a role in the receptivity of the endometrium (Poncelet, Cornelis et al. 2010). N-cadherin was found to be one of the Wnt associated genes in a profiling study (Nguyen, Sprung et al. 2012) and, given the known importance of Wnt signalling in stem cell biology, N-cadherin was investigated to determine if it could be a potential marker of the endometrial epithelial progenitors (Nguyen, Xiao et al. 2017). N-cadhein protein was shown to be expressed in 16.7% (range 3.7-36.7%) of Epithelial cell adhesion molecule (EpCAM)$^+$ sorted endometrial epithelial cells, similar to when magnetic beads were used to sort (20.2% N-cadherin positive, range 8-35.5%). When colony forming assays were utilised to assess enrichment of epithelial progenitors, larger clones, and significantly higher median cloning efficiency, were observed in the N-cadherin$^+$ cells. These clones were large and densely packed, with small cytokeratin-positive cells, and a high nuclear:cytoplasmic ratio. When serial cloning was undertaken, N-cadherin$^+$ cells generated clones from freshly isolated suspensions, samples underwent up to three rounds of serial cloning, and also differentiated into cytokeratin$^+$ glandlike epithelial structures with a lumen in 3D Matrigel. N-cadherin was found, by IF, to be strongest in the basalis glands adjacent to the myometrium, and rarely co-localised with Ki-67, indicating a quiescent phenotype. SSEA-1 cells were described to be phenotypically distinct from N-cadherin, suggesting a potential epithelial hierarchy (Nguyen, Xiao et al. 2017). Two further IHC studies were also published in the same year. The first compared infertile patients with fibroids, to fertile controls, using IHC and qRT-PCR (Makker, Goel et al. 2017). This showed that N-cadherin was lower in the LE in the mid secretory endometrium of infertile women when compared to fertile controls, but no significant change was demonstrated in

either the immunoexpression or the mRNA. The IHC staining demonstrated by Makker et al (LE expresses strongest levels) (Makker, Goel et al. 2017) is in stark contrast to the IF staining presented in the manuscript by Nguyen et al (basalis adjacent to the myometrium expresses strongest levels) (Nguyen, Xiao et al. 2017). Using purely IHC, Xie and colleagues compared N-cadherin between patients with endometriod adenocarcinoma, and normal controls (Xie, Zheng et al. 2017). They showed that N-cadherin was positive when brown/yellow particles were seen in the cytoplasm of a cell (again contradictory to other published studies). For the 50 normal samples that were reported on in this study, the positive expression rate for N-cadherin protein was 40.0% (8 weak positive, 9 moderate positive, and 3 strong positive) and the positive N-cadherin protein expression rate was statistically higher in the endometriod adenocarcinoma group compared to the normal controls ($p < 0.05$). They also showed that E-cadherin is commonly not expressed in the cells with positive N-cadherin expression, therefore, a transition may exist between them (Xie, Zheng et al. 2017).

The published studies on endometrial N-cadherin seem to report major conflicting differences. However, these studies have reported on N-cadherin expression in a variety of patient populations (healthy and pathological), using different techniques; the exact clones identified by different anti-N-cadherin antibodies were inconsistent and this makes it difficult to draw conclusions from the available N-cadherin data. Although the N-cadherin expressing cells may have some progenitor activity, their exact position in the human endometrial epithelial differentiation hierarchy is yet to be confirmed.

1.5.2.1.2.3 Stromal stem cell markers

Stromal ASC work was initiated with identifying markers preferentially expressed in the cell populations demonstrating higher clonogenic properties *in vitro,* by Gargett and her colleagues.

1.5.2.1.2.3.1 Co-expression of CD146 and platelet derived growth factor–receptor β

The first markers proposed to identify an endometrial stromal ASC population were CD146, and platelet derived growth factor–receptor β (PDGF-Rβ) (2 perivascular cell markers). This is because their co-expression was detected in cells with higher clonogenic ability *in vitro*. These cells were located in the perivascular area in both the functionalis, and the basalis of the intact full thickness human endometrium (Schwab and Gargett 2007). *In vitro* FACS sorted $CD146^{+}$/PDGF-$R\beta^{+}$ cells had significantly greater colony-forming capacities than $CD146^{-}$/PDGF-$R\beta^{-}$ cell populations (7.7 ± 1.7% versus 0.7 ± 0.2% respectively) (p=0.0001) (Schwab and Gargett 2007). $CD146^{+}$/ PDGF-$R\beta^{+}$ cells produced more large colonies with densely packed cells and a high nuclear:cytoplasmic ratio. The $CD146^{+}$PDGF-$R\beta^{+}$ cells expressed typical MSC surface markers such as CD29, CD44, CD73, CD90 and CD105, and were negative for haematopoietic (CD34, CD45), and endothelial markers (CD31) (Schwab and Gargett 2007). When cultured in appropriate induction media, the $CD146^{+}$PDGF-$R\beta^{+}$ cells underwent multilineage mesenchymal differentiation into adipogenic, myogenic, chondrogenic, and osteoblastic lineages (Schwab and Gargett 2007). These studies used pooled, clonally derived $CD146^{+}$PDGF-$R\beta^{+}$ cells (not singly expanded clones) as well as not using a positive control of known multi-lineage differentiation (such as MSCs), thus making it difficult to determine their true differentiation ability. This study did not consider the $CD146^{+}$PDGF-$R\beta^{+}$ cells any further after colony forming capacity, and multi-lineage differentiation ability, therefore, we are not able to comment on their capacity to produce endometrial stroma in 3D culture, or *in vivo* tissue reconstitution ability. Without this evidence, it is difficult to conclude that these isolated cells are in fact an ASC population. It is thought that the $CD146^{+}$PDGF-$R\beta^{+}$ subpopulation are similar to bone marrow and adipose tissue MSCs in their differentiation potential, and in their perivascular location identified for MSC in many organs (Crisan, Yap et al. 2008).

1.5.2.1.2.3.2 W5C5/ SUSD2

W5C5 was the next ASC marker reported in the endometrial stroma, and it was particularly successful in selecting endometrial MSC. $W5C5^+$ cells represented 4.2 ± 0.6% of the freshly sorted endometrial stromal cells using flow cytometry, located peri-vascularly in the basalis, and functionalis, with a significantly greater clonogenicity (median 3.6: range, 0.7-6.9) than depleted counterparts (median 0.6: range, 0.1-3.8). $W5C5^+$ cells were able to be differentiated into adipocytes, osteocytes, chondrocytes, myocytes, and endothelial cells; producing endometrial stromal-like tissue *in vivo* (no MSC were used as an external control). $W5C5^+$ cells were transplanted under the kidney capsule of NOD scid gamma (NSG) mice, and white growths (small masses) were identified macroscopically on 2 out of 10 kidneys. When the mice were examined by histological and microscopic analyses, stromal-like connective tissue was revealed under all of the kidney capsules. The $W5C5^+$ cells produced significantly greater numbers of CFUs and the study identified W5C5 as a single marker capable of purifying endometrial MSCs (Masuda, Anwar et al. 2012), thus negating the need to use two markers ($CD146^+$/PDGF-$R\beta^+$).

1.5.2.1.2.3.3 MSCA-1

MSCA-1, a bone marrow-derived MSC surface marker, has been identified to be identical to Tissue Non-specific Alkaline Phosphatase (TNAP) (Sobiesiak, Sivasubramaniyan et al. 2010), which belongs to a large family of dimeric enzymes common to all organisms and expressed in liver, bone, kidney, and endometrium (Bitensky and Cohen 1965, Wilson 1969, Wilson 1976, Hoshi, Amizuka et al. 1997) as well as in ESCs (O'Connor, Kardel et al. 2008) (when ESC differentiate the expression of TNAP decreases) (Sobiesiak, Sivasubramaniyan et al. 2010). TNAP is expressed on endometrial perivascular cells, the proposed endometrial MSC-like cell location (Schwab and Gargett 2007). The proportion of $W8B2^+CD146^+$ endometrial stromal cells was compared to the proportion of $CD146^+PDGFR\beta^+$ MSC-like cells found in human endometrium, and these were very similar, leading to the conclusion that endometrial MSC-like cells express TNAP. Combined with

CD146, this ectoenzyme was proposed to be a suitable marker for the isolation from the $EpCAM^-$ endometrial stromal population. Due to TNAP being exclusively expressed in the $CD146^+$ subset, but not on other MSC-like/fibroblast-like cells, it would appear that TNAP is developmentally expressed on MSC/pericyte progenitor cells, and is down-regulated during further differentiation.

Not only was TNAP expressed on the endometrial perivascular cells, but it also stained endometrial epithelial tissue on the apical luminal surface. This has led to TNAP also being proposed as a marker for the isolation of a subset of endometrial glandular epithelial cells. The fact that TNAP is found in two separate niches in the endometrium makes this potential MSC marker confusing, and if studies were to be completed showing that the TNAP cells could recapitulate endometrial tissue in animal models, and had multilineage potential, then the cells would need to be sorted on TNAP, and also EPCAM, therefore, TNAP is not suitable as a single marker isolation protocol for endometrial MSC. However, the fact it is expressed in cells of the stromal and epithelial compartment is interesting, and if further studies demonstrate that cells expressing TNAP from both fractions possess stem cell characteristics, it may be a common marker for cells involved in the endometrial regeneration process, and a good therapeutic target. Consequently, further studies are warranted in this area.

1.5.2.1.2.4 Epithelial stem cell markers

Unlike the stromal studies, the study of epithelial stem cell markers considered the hypothesis that the stem cells should reside in the basalis glands, or in the PM epithelium. The basalis markers described above (SSEA-1, nuclear SOX9) were the first basalis markers to be presented as epithelial ASC markers, in 2013. The recent work also suggests N-cadherin to be another basalis marker. These studies, however, are based on the presumption that human endometrial glandular architectural arrangement is a single blunt ended tube, which is yet to be confirmed in 3D reconstruction. Particularly the latter study suggests; the deeper in the glandular base the cells are, the more likely they are to be marking the more primitive cell, i.e. a

hierarchical arrangement depending on the location within the presumed single tubular, blind-ended structure (Nguyen, Xiao et al. 2017). Hence, the localisation of these need to be re-examined with the 3D architectural re-modelling of the endometrial glands.

1.5.2.2 *In Vitro* Functional studies

1.5.2.2.1 Colony forming units, clonogenicity

In 2004, clonogenicity (the ability of a single cell to form colonies, a key feature of stem cells) of endometrial derived cells was demonstrated, this suggested a definitive stem cell phenotype (Chan, Schwab et al. 2004). Epithelial and stromal cells from the endometrium were seeded at clonal density, and cloning efficiency (CE) was assessed *in vitro*. The resulting colonies of endometrial cells were of two types; one large tightly-packed, and one, more common, smaller dense type. Endometrial epithelial colony forming units (CFU) could be cultured in a serum-free medium, containing EGF, or transforming growth factor-α, this suggested that they express EGF receptors (Chan, Schwab et al. 2004, Schwab, Chan et al. 2005, Gargett 2006). The stem cell niche was shown to be important to endometrial epithelial progenitor cell activity, as mouse fibroblast feeder layers were required for serum-free clonal culture. The CE of epithelia was 0.22 ± 0.07%, and stroma had an overall CE of 1.25 ± 0.18%. The smaller colonies were thought to represent transient amplifiers (TAs); they were more common and had limited self-renewal. The larger colonies were assumed to represent primitive ASCs; they were less common and more proliferative. This proposal is not fully consistent with progenitor cells having higher proliferative activity and primitive stem cells being more quiescent. Furthermore, stem cells (particularly epithelial) are likely to differentiate rapidly when removed from their niche, therefore, they may not necessarily produce larger colonies in *in vitro* culture after tissue disruption and isolation in to single cells.

Follow-up studies on epithelial CFU indicated that individual large CFU had substantial self-renewal activity when seeded at very low density (10 cells/cm^2), compared to small CFUs (large CFU subcloning 2.9 times vs small CFU 0.5 times) (Gargett, Schwab et al. 2009). When cultured in

Matrigel the large epithelial CFU differentiated into large cytokeratin-expressing gland-like structures, although the low power phase micrographs of these structures grown on 3D matrix presented in this particular manuscript is less persuasive of the claim (Gargett, Schwab et al. 2009).

This work concluded that endometrial epithelial progenitors were represented in the large CFU with the ability to self-renew and differentiate *in vitro,* and the small CFU potentially to be initiated by more mature TA cells.

1.5.2.2.2 Side population

SP cells (0–5%) have been identified in fresh isolates (Tsuji, Yoshimoto et al. 2008, Cervello and Simon 2009, Masuda, Matsuzaki et al. 2010) and short-term cultures (Kato, Yoshimoto et al. 2007) of human endometrial cells. The percentage of SP cells in single cell endometrial suspensions derived from different patient samples were reported to be highly variable between individuals. However, higher numbers seemed to be found in the menstrual (Kato, Yoshimoto et al. 2007) and proliferative (Tsuji, Yoshimoto et al. 2008, Masuda, Matsuzaki et al. 2010) stages of the cycle. This percentage variability was also mirrored by CFU activity in human endometrium. There is no consensus as to whether the absolute number of SP cells are stable throughout the menstrual cycle or not, in agreement with the concept that ASCs, being a small, quiescent and static, resident population (Masuda, Matsuzaki et al. 2010), the decline of SPs in the secretory phase may result from dilution as the functionalis grows, and increases in thickness. When SP cells (freshly sorted) showed little growth in culture, the authors argued that this was secondary to them being quiescent (most of the cells (85%), being in G0 phase of the cell cycle). This is a proposed feature of ASCs, but, in contrast, the SP cells sorted from endometrial short-term cultures were, primarily, in G1 and G1/M/S phases (Tsuji, Yoshimoto et al. 2008). Endometrial SP cells sorted from short-term cultures did not express endometrial epithelial (CD9), or stromal (CD13) cell differentiation markers to start with, but these markers were re-expressed in subsequent long-term Matrigel cultures, indicating a capacity to differentiate into $CD9^{+}$E-cadherin^{+} gland-like organoids (suggesting epithelial differentiation), and

$CD13^+$ stromal clusters, when cultured for a further 2 months on collagen coated dishes (Kato, Yoshimoto et al. 2007). However, even FACS, the most efficient sorting method available, does not have 100% efficiency. Therefore, particularly in long-term culture, the small percentage of both epithelial and stromal cells that are likely to have been contaminating the initial SP population (although the authors reported that they do not express epithelial/stromal markers) are likely to have expanded. The other reports examining the endometrial SP show SP cells in both the stromal, and epithelial population (Cervello, Gil-Sanchis et al. 2010, Masuda, Matsuzaki et al. 2010, Cervello, Mas et al. 2011). Furthermore, it is known that endometrial cells in 2D culture lose their phenotypical markers and undergo culture related changes, thus, cultured cells may have lost their markers, but regain them in the more physiologically relevant growth environment; the 3D matrix. Finally, the achievement of single cell suspension from the solid tissue samples that is necessary for cell sorting (e.g. enzymatic digestion) will remove the cell surface proteins, which will later be re-formed in cells with prolonged culture. All these issues needed to be considered when the current literature was interpreted. The sorted short-term cultured SP cells were able to be maintained in culture for 3 months, whereas the non-SP cells sorted from the short term cultured SP cells became senescent within 3 months (Kato, Yoshimoto et al. 2007). This evidence agrees with the longevity associated with ASCs as opposed to differentiated cells.

To demonstrate their functional potential, cultured endometrial SP cells were shown to decidualise after treating with oestradiol and progesterone, therefore confirming their ability to assume the morphologic, and functional, changes characteristic of the secretory endometrium (Tsuji, Yoshimoto et al. 2008).

Additionally, by differentiating into adipocytes and osteoblasts *in vitro,* endometrial epithelial SP cells have been demonstrated to be multipotent *(Cervello, Gil-Sanchis et al. 2010).* After 2 weeks in culture, the authors reported the presence of Oil Red O lipid vacuoles in adipogenic induction media (but the round morphology typical of adipocytes was not seen). Similarly, in osteogenic induction media, positive immuno-reactivity for bone

sialoprotein was reported. Once again, this evidence is intriguing, and needs to be considered with the possibility of contamination with stromal cells (which are known to have the ability to differentiate in the mesenchymal lineages). Further evidence has been produced by Kurita et al., with their elegant set of xenograft experiments, demonstrating the adult endometrial epithelium to be lineage specific (Kurita, Cooke et al. 2001).

Masuda et al., demonstrated unipotency of the endometrial epithelial SP cells *in vitro* by injecting them under the mouse kidney capsule (mesodermal derivative), and generating endometrial tissue; those cells did not differentiate into kidney parenchyma (Masuda, Matsuzaki et al. 2010). This unipotency was echoed with work showing that SSEA-1 positive cells grown in a chimeric explant model, using kidney tissue isolated from CD1 neonatal mice, injected under the kidney capsule, produced endometrial gland like structures staining positive for endometrial differentiation markers (Matthew 2013).

Serum oestradiol levels have been shown to change in the same manner as the proportion of SP cells in postpartum mice, perhaps indicating that oestrogen is a prerequisite for increasing SP cells (Xu, Hu et al. 2011). This finding of increased oestrogen was confirmed within a mouse endometrial injury model, stromal SP cells significantly increased 6 hours after injury, but they were dependent on oestrogen, not progesterone or a combination of oestrogen and progesterone (Hyodo, Matsubara et al. 2011).

1.5.2.3 *In vitro* studies utilising functional assays

1.5.2.3.1 Differentiation

Differentiation potential, the ability of a stem cell to produce differentiated progeny, is a stem cell property. Most work assessing multipotency of endometrial cells has focused on the stromal population, due to the greater abundance in endometrial tissue, and difficulties with epithelial culture. Furthermore, the tissue reconstruction studies have shown interesting data on endometrial cellular plasticity. The endometrial epithelial phenotype is driven by the stromal component in the neonatal mice (Cunha 1976, Kurita,

Cooke et al. 2001), but in tissue reconstruction experiments the authors have shown that uterine epithelial cells from adult mice, when transplanted with mesenchymal cells of adult vagina, did not change their original simple columnar uterine phenotype. After 1 month of *in vivo* growth, adult uterine epithelial cells, recombined with vaginal mesenchymal cells, remained simple columnar. Thus, differentiation of adult uterine epithelium was unresponsive to induction by vaginal mesenchyme (Kurita, Cooke et al. 2001).

In adult tissue, epithelium drives the phenotype despite the stroma, suggesting that stroma retains the plasticity and epithelia does not. Therefore, the epithelium should be the driving force in endometrial regeneration.

1.5.2.3.2 Menstrual blood stem cells

In 2007, menstrual blood was used to obtain endometrial stromal ASCs, and these were capable of differentiation into adipocytes, osteoblasts, chondrocytes, cardiocytes, myocytes, and endothelia. These menstrual blood stromal ASCs were also capable of trans-differentiation into endodermal and ectodermal tissue, such as hepatocytes, pulmonary epithelia, and neurones. The ASCs were mononuclear, and demonstrated positive immuno-reactivity for CD90, CD73, and CD103, but were devoid of CD34, and CD45, suggesting the cells are of mesenchymal, not haematopoietic origin (Meng, Ichim et al. 2007). Menstrual blood derived stem cells are an attractive target as a treatment for many diseases, secondary to their ease of collection and autologous nature (Borlongan, Kaneko et al. 2010). The use of autologous cells for the subacute phase of stroke offers practical clinical application (Kaneko, Dailey et al. 2013). When grown in appropriate conditioned media, they express neuronal phenotypic markers (Nestin, MAP2), and in an *in vitro* stroke model of oxygen glucose deprivation, it was found that oxygen glucose deprived-exposed primary rat neurons, that were co-cultured with menstrual blood-derived stem cells, or exposed to the media collected from cultured menstrual blood, exhibited significantly reduced cell death. Transplantation of menstrual blood-derived stem cells (either intracerebrally, or intravenously, and without

immunosuppression) into a rat model of ischaemic stroke, significantly reduced behavioural and histological impairments, compared to vehicle-infused rats, supporting the use of menstrual blood-derived cells as a stem cell source for cell therapy in stroke (Borlongan, Kaneko et al. 2010, Rodrigues, Glover et al. 2011, Kaneko, Dailey et al. 2013, Azedi, Kazemnejad et al. 2014) and other basal ganglia disorders, such as Parkinson`s and Huntington`s disease (Rodrigues, Voltarelli et al. 2012).

Sepsis (in the cecal ligation and puncture mouse model) has also shown to have improved outcomes when menstrual derived stem cells are utilised in the treatment regime, alongside antibiotics. Menstrual derived stem cells, in synergy with antibiotics, improved the survival rate (95%) in comparison; with saline (6%); antibiotics alone (73%); and menstrual derived stem cells alone (48%); concluding that menstrual derived stem cells could constitute a feasible approach for the future clinical treatment of sepsis (Alcayaga-Miranda, Cuenca et al. 2015).

A mouse model of premature ovarian failure, treated with menstrual blood stem cells, expressed higher levels of ovarian markers (AMH, inhibin α/β and FSH receptor), and the proliferative marker Ki67. In addition, the overall weight, plasma oestrogen level, and number of normal follicles increased overtime compared with controls (Liu, Huang et al. 2014).

Menstrual blood stem cells have been differentiated into hepatocyte-like cells, and demonstrated *in vitro* mature hepatocyte functions such as urea synthesis, glycogen storage, and indocyanine green uptake; leading to their potential to be used in chronic liver disease (Mou, Lin et al. 2013, Khanjani, Khanmohammadi et al. 2014, Khanjani, Khanmohammadi et al. 2015).

The type 1 diabetes mellitus mouse model was used to show the therapeutic effects of menstrual blood stem cells on the mechanism of β-cell regeneration after transplantation. The menstrual blood stem cells reversed hyperglycaemia and weight loss, prolonged lifespan, and increased insulin production in the diabetic mice. The mice recovered islet structures and increased their β-cell number, with the majority of the menstrual blood stem cells migrating into the damaged pancreas, and being located at the

islet, duct, and exocrine tissue. The menstrual blood derived stem cells were found to enhance neurogenin3 expression (represents endocrine progenitors), rather than differentiate into insulin-producing cells, concluding that they stimulated β-cell regeneration through promoting differentiation of endogenous progenitor cells (Wu, Luo et al. 2014).

Menstrual blood derived stem cells have also been proposed to be used for; bone tissue-engineering purposes (taking advantage of their osteogenic driving potential) (Darzi, Zarnani et al. 2012); dermatological lesions and diseases (Faramarzi, Mehrabani et al. 2016); heart muscle repair (Hida, Nishiyama et al. 2008); limb ischaemia (Murphy, Wang et al. 2008); and muscular dystrophy (Cui, Uyama et al. 2007).

More recently it has been shown that these MSCs derived from menstrual blood have the ability to secrete decidualisation markers (prolactin and insulin-like growth factor binding protein-1), and differentiate into decidualised cells, leading to the potential of a therapy for decidualisation insufficiency (Domnina, Novikova et al. 2015). When cultured in differentiation-inducing media supplemented with 20% human follicular fluid, the human menstrual blood stem cells form oocyte-like cells and express germ cell markers. Cells also expressed FSH and LH receptors, and produced oestrogen and progesterone regulated by gonadotrophin suggesting a potential to differentiate in to ovarian tissue (Lai, Guo et al. 2016). The exact origin of these cells (endometrial or bone marrow derived) however, is not known.

1.5.2.3.3 Differentiation potential of endometrial stromal stem cells

In 2009, endometrial stromal ASCs were shown to be capable of multi-lineage differentiation into fat, bone (confirmed with presence of osteopontin, ostenectin and alkaline phosphatase) (Azami, Ai et al. 2013), cartilage, skeletal muscle (Gargett, Schwab et al. 2009, Fayazi, Salehnia et al. 2015), and smooth muscle (expressing specific smooth muscle cell markers including α-smooth muscle actin, desmin, vinculin and calponin) (Shoae-Hassani, Sharif et al. 2013). Plasticity has also been shown by the trans-differentiation of endometrial stromal ASCs into neural (neural and glial

lineage markers such as Nestin, NF-L, MAP2, PDGFRa, CNP, Olig2, MBP and GFAP) (Ebrahimi-Barough, Hoveizi et al. 2015, Fayazi, Salehnia et al. 2015), Schwann cells (expression of S100 and P75 noted) (Bayat, Ebrahimi-Barough et al. 2016), Oligodendrocytes (Ebrahimi-Barough, Kouchesfahani et al. 2013), pancreatic cells (shown by secretion of insulin and markers of β cells such as PDX1, proinsulin and c-peptide) (Santamaria, Massasa et al. 2011, Niknamasl, Ostad et al. 2014), urinary bladder epithelial cells (urothelium, as tested by urothelium-specific genes and proteins, uroplakin-Ia/Ib, II, III and cytokeratin 20) (Shoae-Hassani, Mortazavi-Tabatabaei et al. 2015), hepatocytes (biomarkers albumin and cytokeratin 8, reduced alpha-fetoprotein and alpha smooth muscle actin expression, synthesised urea, and stored glycogen) (Snykers, De Kock et al. 2009, Yang, Wang et al. 2014), and megakaryocytes (identified by expression of CD41a and CD42b and reduction of pluripotent transcription factors Oct4 and Sox2, platelets were seen and functional as evidenced by the up regulation of CD62p expression and fibrinogen binding following thrombin stimulation); both *in vitro,* and in animal models (Wang, Chen et al. 2012, Khademi, Soleimani et al. 2014).

Animal models of Duchenne muscular disease (Toyoda, Cui et al. 2007), stroke (Allickson, Sanchez et al. 2011), diabetes (Wu, Luo et al. 2014), Parkinson`s disease (Wolff, Gao et al. 2011, Wolff, Mutlu et al. 2015), and critical limb ischemia (Murphy, Wang et al. 2008) suggest endometrial stromal ASCs improve outcomes.

1.5.2.3.4 Differentiation potential of endometrial epithelial stem cells

Unipotency was shown in epithelial cells in 2009 by differentiating endometrial epithelial ASCs into CK^+ gland-like structures, in 3D Matrigel (Gargett, Schwab et al. 2009). An assessment of multipotency was not possible as epithelial cells were unable to grow to confluency. Intriguingly, as mentioned earlier, endometrial epithelial SP cells have been demonstrated to be multipotent by differentiating into adipocytes and osteoblasts *in vitro* (Cervello, Gil-Sanchis et al. 2010). Adult human endometrial epithelial cells are expected to be committed to an endometrial phenotype, therefore, they

are presumed to not have the potential for differentiating in to other lineages. In our laboratory, the kidney-endometrium human-mouse chimera, supported growth of kidney structures from mouse cells, and endometrial gland like structures by human endometrial epithelium demonstrated uni-potency of these cells (Mathew, Drury et al. 2016).

1.5.2.3.5 Label retaining cells

Locating LRC in animal subjects (as BrdU is a recognised health hazard, and the use of the LRC technique in humans is not permitted) is a method of identifying somatic stem/progenitor cells and their location in the stem cell niche when specific markers are unknown. This method relies on the infrequent cell turnover of most ASCs, in comparison to rapidly proliferating TA cells (Chan and Gargett 2006, Gargett 2007). Mouse endometrium was pulse labelled with BrdU and studied after an 8-week chase to identify endometrial LRC.

1.5.2.3.5.1 Epithelial LRCs

3% of the epithelial nuclei were $BrdU^+$and were located in the LE. They were shown to be negative for ERα through dual labelling IF, providing evidence that LE stem/progenitor cells are responsible for the growth of glands during development and in cycling mice (Chan and Gargett 2006). In ovariectomised prepubertal mice the first cells to proliferate in oestrogen-stimulated endometrial growth differed from ovariectomised cycling mice, in the first, the epithelial LRC proliferated, suggesting they function as stem/progenitor cells to initiate epithelial regeneration; while in the second epithelial LRC and non-LRC rapidly proliferated to regenerate LE and glandular epithelium (Chan and Gargett 2006). Using a mouse model with menstrual breakdown and repair, ERα negative glandular epithelial LRC contributed to repair of the LE, following menstruation, post progesterone withdrawal (Chan, Kaitu'u-Lino et al. 2012). Endometrial repair occurred in the absences of oestrogen (Chan, Kaitu'u-Lino et al. 2012). $BrdU^+$ epithelia was lost soon into the chase period, leading to the thoughts that the epithelial regeneration could be relying on the self-duplication of a mature epithelial cell type, or that the LRC technique is not sensitive enough to label

endometrial epithelial cells with an ASC phenotype (Cervello, Martinez-Conejero et al. 2007).

1.5.2.3.5.2 Stromal LRCs

Between 6-9% of the stroma were LRCs located just below the LE, at the endo-myometrial junctions, or near blood vessels (Chan and Gargett 2006, Cervello, Martinez-Conejero et al. 2007), with 84% of them being ERα negative. The cells were found to not be leucocytes (CD45 staining), or endothelia (CD31 staining) (Chan and Gargett 2006). $BrdU^{+}$ cells surrounding blood vessels were positive for alpha smooth muscle actin (α-SMA) making it probable that these cells represent pericytes. In some studies, 0.6% of stromal LRCs co-expressed OCT4 (a pluripotency marker) and c-kit (a haemopoietic stem cell marker) (Cervello, Martinez-Conejero et al. 2007), so they were potentially in an undifferentiated state; but in others neither Sca-1 (Chan and Gargett 2006) nor c-kit (Szotek, Chang et al. 2007) were expressed.

Oestrogen was shown to drive epithelial LRC proliferation in juvenile development, but had a minimal role in epithelial and stromal LRC cyclical regeneration, perhaps indicating that $BrdU^{-}/ER\alpha^{+}$ cells release paracrine factors to mediate a LRC response (Chan, Kaitu'u-Lino et al. 2012).

A relationship between stromal and epithelial ASCs was seen when stromal SP cells were transplanted under the kidney capsule in mice. Only endometrial stroma was formed, and when epithelial SP cells were transplanted, only epithelia was formed. Endometrial-like tissue was only generated when both these populations were combined. This would suggest two distinct ASCs exist; a stromal and an epithelial ASC (Cervello, Mas et al. 2011).

1.5.2.3.6 Bone marrow as a source of endometrial ASCs

Bone marrow, as a source of endometrial regeneration, is supported by the ability of bone marrow derived MSC to produce ‘decidua-like’ stroma, after activation of the PKA pathway *in vitro* (Aghajanova, Horcajadas et al. 2010); together with bone marrow derived cells being found in the decidua of normal

murine pregnancy (Lysiak and Lala 1992). Co-culture of bone marrow derived cells with endometrial stromal cells, and oestrogen stimulation, resulted in CK^+ endometrial epithelial like cells (Zhang, Cheng et al. 2012).

When male bone marrow derived cells were transplanted into female mice, and Fluorescence in situ hybridisation of the mice endometrium was completed, Y-chromosomes were present in a tiny 0.0002% of CD45-/F4/80- epithelial cells, and 0.0003% of CD45-/F4/80- stromal cells (Du and Taylor 2007). In another set of experiments, samples from female mice harvested 40 days after a haematological stem cell transplant, showed that 6% average donor-derived ECs were detected, concluding that bone marrow-derived endothelial progenitors contribute to the formation of new blood vessels in the endometrium (Mints, Jansson et al. 2008).

In a study of Human leucocyte antigen mismatch transplant, donor-derived endometrial cells were detected in endometrial biopsy samples from all bone marrow recipients, and accounted for a wide range (0.2% to 48%) of epithelial (displaying CD9 marker), and stromal cells (0.3% to 52%) (vimentin positive) (Taylor 2004, Ikoma, Kyo et al. 2009, Cervello, Gil-Sanchis et al. 2012). SP cells were not shown to be formed by XY donor-derived cells (Cervello, Gil-Sanchis et al. 2012).

Therefore, the evidence presented above may suggest bone marrow to also be a source of endometrial regeneration, but its contribution seems to be low. It is likely that bone marrow could be implicated in endometrial repair after times of massive injury, such as ablation, and in the formation of the decidua; when the endometrium requires ‘extra-assistance’.

In a much more recent study, authors were characterising bone marrow derived cells in the endometrium of mice transplant recipients, and found no evidence of bone marrow derived stroma, epithelium or endothelium. All of the cells that were detected in the endometrium were immune cells expressing the pan-leukocyte marker CD45, including $CD3^+$ T cells and $F4/80^+$ macrophages; immuno-stained weakly for CD45. The macrophages were abundant in the stroma, infiltrated the epithelial and vascular compartments, and were documented that they could easily be mistaken for

bone marrow derived endometrial cells. The authors concluded (in disagreement with previous studies) that bone marrow cells are unlikely to transdifferentiate into endometrial stroma, epithelium and endothelium. They warned of the massive implications, as expectations for bone marrow derived endometrial stem cells have been high, with numerous treatment strategies discussed previously (Ong, Cousins et al. 2018).

1.5.2.4 Direct confirmation of the existence of endometrial epithelial progenitor cells inferred from gland methylation patterns

Methylation patterns (to represent random replication errors) have been used to reconstruct endometrial epithelial cell lineages; with the hypothesis that all daughter cells are originating from a common ancestor, and cell divisions and ancestry may be surreptitiously recorded by identifying replication errors that naturally accumulate in a clock-like manner during aging (increased with age), parity (decreased by parity), or BMI (increased with increased BMI) (Pike, Pearce et al. 2004, Kim, Tavare et al. 2005).

In 2003, Tanaka et al used a collagenase based method to isolate endometrial glands from the stroma, and extract DNA from individual endometrial glands; to examine them for chromosome inactivation patterns and determine the clonal constitution of glandular cells, and the LE, in order to discuss the presence of endometrial stem cells, as well as endometrial carcinogenesis (Tanaka, Kyo et al. 2003). PCR amplification of the CAG repeats in the androgen receptor (AR) gene were utilised to assess the clonality of the endometrial epithelial cells. The AR linked to the X chromosome has a polymorphic short tandem repeat of the trinucleotide [CAG]n. 90% of the general population are heterozygous for the length of CAG repeats (Allen, Zoghbi et al. 1992, Enomoto, Fujita et al. 1994). Methylation of the HhaI endonuclease sites near the CAG repeats correlates with X chromosome inactivation (Wolf and Migeon 1982, Allen, Zoghbi et al. 1992). Digesting samples with the enzyme Hhal generated homogenous products derived from either the paternal, or maternal, inactive X

chromosome (HhaI can cut the unmethylated sites on an active X chromosome, but cannot cut the methylated sited on an inactive X chromosome) (Tanaka, Kyo et al. 2003). The results of this experiment, in 7 of the 10 patients (3 were excluded as the size of the amplification products did not differ between the two alleles), showed that DNA recovered from almost all of the glands, from each patient, yielded two distinct PCR bands, in the absence of HhaI digestion. After HhaI digestion, the DNA yielded a single band, indicating the monoclonal cellular composition of the glands. To examine if the monoclonal composition is limited to a single gland, or extends to the surrounding glands, an endometrial surface area of 25 mm^2 was surgically extracted, subdivided, and glands were isolated from each piece for DNA extraction and analysis of clonality. In 15 of the 25 pieces, all 10 glands isolated from each piece exhibited the same clonality, suggesting that glands within a 1mm^2 area share clonality, and the monoclonal composition of endometrial glands is regionally defined. The remaining 10 pieces contained glands that exhibited clonality of mixed parental origins. The authors are unsure if these pieces contained abutting areas of monoclonal groups or one field with both clones integrated. To test clonality further, a GFP labelled (on either the maternal or paternal X chromosome) mouse model was utilised. Mouse cells that have the transgene on the active X chromosome are fluorescent, whereas those that have the transgene on the inactive X chromosome are not (Hadjantonakis, Gertsenstein et al. 1998, Hadjantonakis, Cox et al. 2001). Individual mouse endometrial glands were either fluorescent or nonfluorescent, demonstrating the monoclonal composition of glandular cells. Interestingly, the LE had clear boundaries between fluorescent and nonfluorescent cell layers, indicating the boundaries where epithelial cells with different clonalities, converge (Tanaka, Kyo et al. 2003).

The authors concluded that the composition of epithelial cells in human endometrium is monoclonal, and monoclonal growth is a hallmark of cancer cells. Polyclonal growth is exhibited by normal cells, this signifies that endometrial glands must be an exception to the theory and poses the suggestion that maybe, secondary to the endometrium being continuously

renewing, the cells must be capable of long-term self-maintenance, with the properties of stem cells (Tanaka, Kyo et al. 2003). The authors postulated that it is likely that stem cells with the same clonality are located in the basal layers in each gland, and, therefore, when endometrial cancer develops, this would be from the stem cells at the base of the glands. They concluded that further analysis is needed to clarify the number of stem cells, and the existence of niches, in endometrial glands (Tanaka, Kyo et al. 2003). The main criticism of this work is that the method used to extract the epithelial glands from the stroma is a method developed for the colon, where the glands are blind ending tubes, and have a definitive end. The method, and authors, presume that human endometrial glandular architecture is the same as the intestine; single tubular architecture with a blind-ended tube. As described in the results section of this thesis, this presumption is erroneous. Therefore, the same method utilised on the human endometrium could have prevented the authors from extracting the whole endometrial glands, which are much more complex than the intestinal crypts (tortuous functionalis portion of the endometrial glands, and branching interconnecting basalis architecture of endometrial glands (Chapter 4) are rather differently arranged). This previous study is unlikely to have identified the contribution of the glandular bases to their clonality assessment.

In 2005, Kim et al tracked methylation patterns to assess stem cell progeny. The glands were freed from the stroma again with an isolation procedure developed for the colon (Potten, Kellett et al. 1992), thus, for the aforementioned reasons, the method is unlikely to have isolated all portions of the endometrial epithelial fraction, gathering just the functionalis glandular portion. The authors proposed methylation patterns in the endometrium, to follow the hypothesis that methylation increased after menarche to menopause, thereafter, levels were relatively stable, indicating that the number of epigenetic marks was a reflection of the mitotic activity of endometrial stem/progenitor cells (Kim, Tavare et al. 2005). Different methylation patterns were found within the same uterus, this would be consistent with individual glands being maintained by distinct stem cell niches that evolve independently (Kim, Tavare et al. 2005). Mathematical

predictive analysis indicated that individual glands contain stem cell niches occupied by several long-lived stem cells. Symmetric and asymmetric cell divisions occurred in a stochastic manner, maintaining a constant number of ASCs in the endometrial gland niche (Kim, Tavare et al. 2005), and arguing against the monoclonality concept that had been previously proposed (Tanaka, Kyo et al. 2003). Once again, this method, utilised a gland extraction method that is not appropriate for the endometrial glands, but to the crypts of the colon, therefore it is not sufficient to conclude on clonality, or stem cell arrangement, of the human endometrial epithelium.

Chapter 2. Methods

2.1 Ethical Approval

Ethical approval for the study was obtained from Liverpool Adult Research Ethics committee (LREC09/H1005/55). Tissue was collected from patients who attended Liverpool Women's hospital (LWH) from 2009-2017 after patients gave written informed consent.

2.2 Collection of human samples

Human endometrial samples were obtained from 119 women undergoing surgery for benign gynaecological conditions (e.g.prolapse), without any evidence of endometrial pathology (Table 2.1).

Sample no.	Age	BMI	Smoker	Parity
1	21	25.9	No	0
2	23	23.5	No	0
3	24	23.8	No	3
4	25	20.9	No	0
5	26	33.4	Yes	2
6	27	23	No	3
7	27	33.7	No	2
8	27	21.1	No	4
9	27	23	No	5
10	30	26.7	No	1
11	31	25.8	Yes	2
12	31	24.9	No	3

13	32	26.6	No	2
14	32	27.8	Yes	2
15	33	26.1	No	2
16	33	27.4	Yes	3
17	33	32.7	Yes	3
18	33	25.9	No	5
19	35	32	No	3
20	35	24.4	No	1
21	35	30.5	No	0
22	35		No	
23	36	23.9	No	4
24	37	22.8	Yes	2
25	37	39.2	No	2
26	37	21.7	No	4
27	37	25.7	No	0
28	39	36.9	No	4
29	39	22.4	Yes	6
30	37	41.5	No	3
31	37	26.5	Yes	2
32	37	30.8	No	2
33	38	30.8	No	3
34	38	27.9	No	4
35	38	26.6	Yes	2

36	39	32	Yes	2
37	39	27.9	No	1
38	39	26.5	No	2
39	40	34.7	Yes	3
40	40	33	No	2
41	40	33	No	2
42	40	27.4	No	
43	41	30.7	No	1
44	41	18.9	Yes	2
45	41	26.7	Yes	4
46	41	23.2	Yes	1
47	42	23.5	No	2
48	42	25.6	Yes	2
49	42	22.3	No	2
50	43	28.6	No	2
51	43	24.5	No	0
52	43	26.7	No	2
53	43	40.5	No	3
54	43	24.2	Yes	2
55	43	25.4	No	2
56	44	24.3	Yes	2
57	44	30.7	Yes	2
58	44	32.3	No	1

59	44	38	No	2
60	44	29.6	No	1
61	44	24.5	No	2
62	44	30.7	No	0
63	44	18.7	No	0
64	44	29.4	No	2
65	44	30.7	Yes	2
66	45	36.4	No	3
67	45	22.5	No	2
68	45	31.6	No	0
69	45	26.1	No	0
70	45	21.7	Yes	4
71	46	23.1	No	3
72	46	25.4	Yes	1
73	46	26.2	No	3
74	46	26.8	No	5
75	47	30.2	No	3
76	47	21.6	No	2
77	47	32.4	No	5
78	47	22.6	No	0
79	47	25	No	1
80	47	23.4	No	1
81	47	25.8	No	2

82	47	29.4	No	2
83	47	24.2	Yes	0
84	47	29.8	No	2
85	48	28.6	No	2
86	48	29.9	No	4
87	48	37.3	No	2
88	48	40.1	No	3
89	49	24.4	No	2
90	49	23.2	No	0
91	49		Yes	2
92	49	28.4	No	2
93	49	27.6	No	3
94	50	20.3	No	3
95	50	21	No	4
96	52	24.5	No	2
97	52	26.3	No	2
98	52	39.6	No	2
99	58	20	No	2
100	60	31	No	2
101	60	26.9	Yes	5
102	61	27.7	No	4
103	62	28.3	No	3
104	61	32.2	No	4

105	62	27.4	No	1
106	65	28.8	Yes	3
107	65	18.5	No	2
108	66	24.9	No	3
109	66	24.9	No	3
110	67	32.8	No	4
111	69	24.7	No	4
112	69	26.8	No	3
113	72	35.8	No	3
114	74	32	No	3
115	74	35.6	No	3
116	75	29.6	No	2
117	76	25.2	No	2
118	77	26.6	No	2
119	78	26.8	No	4

Table 2.1 - Demographical details of the patients involved in the study.

Endometrial and Fallopian tube biopsies were collected, in the theatre, by trained members of the clinical research team, immediately after completion of hysterectomy (full thickness sample), or following gynaecological surgery where the uterus was not removed (pipelle sample). Full thickness “wedge” biopsies from normal pre or post menopausal (PM) endometrium were obtained by cutting a thin slice of endometrium, attached to underlying myometrium, straight after opening the anterior aspect of the uterus, in the coronal plane. Pipelle biopsies were obtained with a suction pipelle (Laboratoire C.C.D., Paris, France) (Figure 2.1 A and B) placed into the endometrial cavity; after gentle scraping, the inner piston of the pipelle was

withdrawn, allowing tissue to be sucked into the pipelle under the effect of vacuum. Fallopian tube biopsies were obtained from the fimbrial end of the fallopian tube, by cutting a slice from the end of one of the Fallopian tubes.

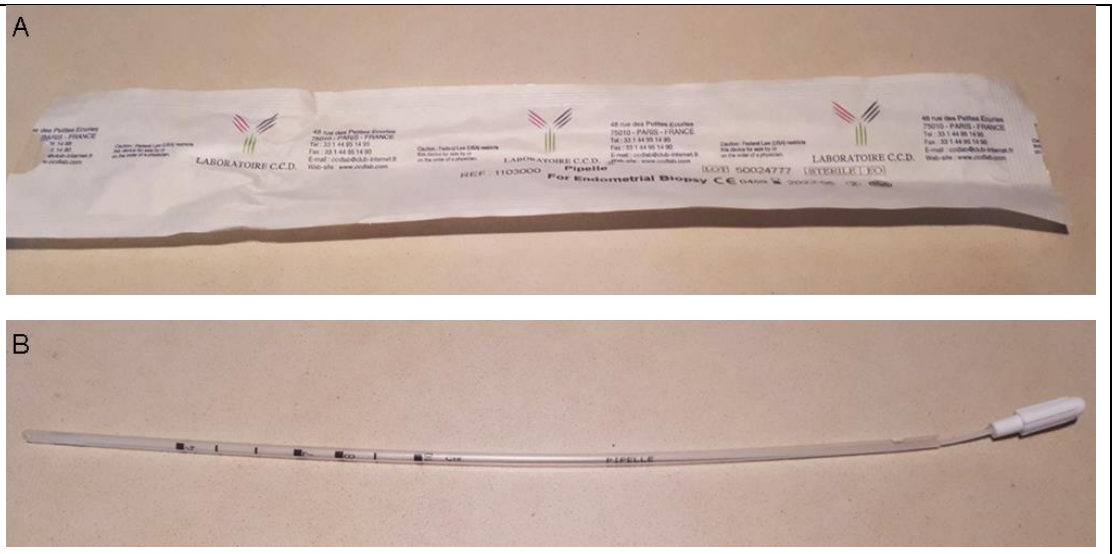


Figure 2.1 (A) – Photograph of **Pipelle**®, flexible polypropylene suction cannula packaging. (B) Photograph of pipelle suction cannula with an outer diameter of 3.1 mm.

Collected samples were split into 3-4 containers, depending on the size of the sample and the experiments to be performed: 15 mL neutral buffered formalin (NBF; Sigma-Aldrich, Dorset, UK), to fix and preserve tissues for subsequent histology processing; 0.5 mL RNAlater (Sigma-Aldrich, Dorset, UK), to permeate the tissue in order to stabilise and protect cellular RNA for subsequent RNA extraction; 15 mL phosphate buffered saline (PBS) (Sigma-Aldrich, Dorset, UK), to remove excess blood prior to snap freezing; and 15 mL Dulbecco's Modified Eagle Medium (DMEMF12) culture medium (Sigma-Aldrich, Dorset, UK), for explant culturing.

2.3 Tissue preparation

2.3.1 Formalin fixed paraffin embedded sample preparations

2.3.1.1 Tissue processing

To avoid autolysis, all samples collected were preserved in 10% NBF directly after collection, and processed after 1-5 days at 4°C in an automated tissue processor (ShandonHistoCentre3, Thermo Fisher, Cheshire, UK). During processing, the tissue samples were dehydrated automatically inside the processor, through increasing concentrations of ethanol, before the cassettes were incubated in xylene (the clearing agent, replacing the dehydrant, with a substance miscible with the paraffin), and impregnated with paraffin (the embedding agent).

2.3.1.2 Tissue embedding

An embedding platform (ShandonHistoCentre3, Thermo Fisher, Cheshire, UK) was used to manually prepare paraffin tissue blocks for sectioning. Following tissue processing, the paraffin impregnated tissue samples were removed from the processing cassettes, orientated in pre-warmed metal block moulds, and fully immersed in paraffin. Any air bubbles trapped in the liquid paraffin were removed by gentle tapping. A fresh tissue cassette was placed on top of the paraffin and, after labelling (specimen number and date), the blocks were left on a cold plate until set, and kept in the freezer (-20°C) overnight (or for at least 30 minutes) prior to sectioning.

2.3.1.3 Tissue Sectioning

The formalin fixed paraffin embedded sample (FFPE) sections (3-4 µm), were cut using a Microtome (Leica RM2255 Rotary Microtome, Leica, Wetziar, Germany), before being floated in a pre-warmed water bath (37°C), left for a few minutes (to remove wrinkles), and collected on 3-Aminopropyltriethoxysilane (APES) coated glass slides (charged slides

provide adhesion for tissue sections), and left to dry at room temperature (RT) overnight.

2.3.1.3.1 RNA free sectioning

When undertaking *In situ* hybridisation (ISH) experiments, FFPE samples were cut in RNase-free conditions to maintain the signal, and decrease background. Prior to sectioning, the microtome and water bath were cleaned with RNASEZAP™ (Sigma-Aldrich, Dorset, UK), the water was changed in the water bath, and the slides were cut onto RNase-free slides (Superfrost, Fisher Scientific, Cheshire, UK). Samples were cut as per sectioning (see above), but dried in a box pre-treated with RNASEZAP™ to minimise contamination.

2.3.2 Frozen tissue processing

Tissue was snap frozen in liquid nitrogen, and stored at -80°C until sectioning was required.

2.3.2.1 Cryo-sectioning

The frozen samples were sectioned at 12 µm onto Membrane Slides 1.0 PEN (D) (Carl Zeiss, Oberkochen, Germany) for enzyme histochemistry and laser capture micro dissection (LCM), in RNase-free conditions, on the Shandon Cryotome FE (Thermo Fisher Scientific, Cheshire, UK). The frozen sections cut for Hematoxylin and Eosin (H&E) staining were sectioned onto 3-Aminopropyltriethoxysilane (APES) coated glass slides slide at 10 µm, before being stored at -80°C prior to staining.

2.3.3 Cycle staging

The premenopausal endometrial tissue specimens were phase typed according to: the patient`s reported last menstrual period (LMP); histological criteria (Noyes, Hertig et al. 1975, Dallenbach-Hellweg 2012) (the H&E section was examined); review of the official pathological report.

2.4 Messenger RNA (mRNA) expression and quantification

2.4.1 RNA isolation

Preliminary isolation of RNA is critical as it determines the reliability of all experimental results performed downstream. The TRIzol/chloroform extraction technique performed, is based on protein denaturation and RNase inactivation by guanidinium isothiocyanate, followed by an acid phenol/chloroform phase separation. Guanidine isothiocyanate was used because it is a chaotropic salt; it destabilises hydrogen bonds, van der Waals forces, and hydrophobic interactions. Proteins, including nucleases, are destabilised, and the association of nucleic acids with water is disrupted, thus allowing optimum conditions for the transfer to silica. The spin cartridges used to isolate RNA contained a clear silica-based membrane, and the RNA in the lysed sample is bound to this membrane. The chaotropic salts enhance and influence the nucleic acid, binding to silica, along with the alcohol. Extracted RNA should be bound to the membrane, and the impurities (protein and polysaccharides) pass through after centrifugation of the lysate. To release the pure RNA from the silica, water was added (the preferred dilutent), RNA readily dissolves in water.

2.4.1.1 Extraction

Total RNA was extracted from frozen and RNA-later endometrial and Fallopian tubes, using the Pure Link RNA mini Kit (Invitrogen LTD., Paisley, UK) following the manufacturer`s protocol.

Firstly, the extraction hood, pipettes, and all other equipment were thoroughly cleaned with 1% Chemgene (Medimark Scientific, Kent, UK), followed by 70% ethanol, and finally RNASEZAP™. The Ultra-Turrax homogeniser (Sigma-Aldrich, Dorset, UK) was assembled, and washing tubes were readied to clean the homogeniser between each sample extraction (5 mL tubes each containing TRIzol or 70% ethanol, one is

required per sample). An RNA free 5 mL bijoux, and a 1.5 mL tube were pre labelled for each sample (sample, name, and date), and the Eppendorf 5424 microcentrifuge (Thermo Fisher Scientific, Loughborough, UK) was pre-cooled to 4°C. SHIELDskin CHEM gloves (Appleton Woods, Birmingham, UK) were worn and changed regularly to protect from the reagents (gloves were changed immediately after handling choloroform, or any spillage, as the break through time was only 4 minutes). The RNA-later samples were homogenised in 1 mL of TRIzol reagent (Thermo Fisher Scientific, Loughborough, UK), with the resulting cell lysate being transferred to the pre labelled 1.5 mL tube, and incubated at RT for 5 minutes, before being placed on ice. Following homogenisation, 200 µL of chloroform (Sigma-Aldrich, Dorset, UK) was added to each sample, and the samples were shaken vigorously for 15 seconds before incubation at RT for 5 minutes. Samples were centrifuged at 12,000 g for 15 minutes at 4°C while spin columns and recovery tubes were appropriately labelled. The upper aqueous phase, containing RNA, was transferred to a new RNAse-free tube, and an equal volume of 70% (v/v) ethanol was added and vortexed.

2.4.1.2 Purification

Approximately 700 µL of the RNA/ethanol solution was transferred onto a Pure Link RNA mini Kit spin column (all was eventually transferred), and centrifuged at 12,000 g, at 18°C for 30 seconds. The flow through was discarded, and the rest of the sample was transferred into the column and spun again at 12,000 g at 18°C for 30 seconds. The RNA binds to the silica based membrane in the spin cartridge during purification. The bound RNA was washed by adding 700 µL of wash buffer I and centrifuging at 12,000 g for 15 seconds at 18°C; followed by two washes and centrifuge spins with 500 µL of wash buffer II to dry the membrane with the attached RNA. The spin column was then transferred to a recovery tube and 30 µl of RNase-free water was added to the centre of the column and left for 1 minute. The sample was then centrifuged for 2 minutes at 18°C at 12,000g, and the column discarded as the RNA had been released from the column membrane and passed in to the recovery tube. 2 µL of the purified RNA was removed from each sample for NanoDrop ND-1000 (Thermo Fisher

Scientific, Loughborough, UK) quantification, and 8 µL was removed for DNase treatment, the remaining purified RNA was stored at -80°C.

2.4.1.3 Quantification

A NanoDrop ND-1000 was used to confirm RNA concentrations and purity spectrophotometrically at the 260/280nm ratio (2 was considered suitable for further experiments).

2.4.1.4 DNase treatment

Prior to transcriptase reactions, to remove any contaminating genomic DNA, 8 µL of purified RNA was treated with 1 µL of DNase I enzyme (NEB, Hertfordshire, UK) in the presence of 1 µL (10×) DNase I reaction buffer (NEB, Hertfordshire, UK), this was incubated for 30 minutes at 42°C. Following this, 1 µL of EGTA (Promega, Hampshire, UK) was added, and a further incubation for 10 minutes at 65°C was performed to stop the reaction.

2.4.1.5 First strand DNA synthesis

RNA needs to be converted to cDNA before PCR can be undertaken. In order to remove secondary structures that could impede long cDNA synthesis, 0.3 -1 µg of purified total RNA was denatured at 70°C for 5 minutes (with 2 µL Oligo (dT) and RNAase free water to make up to 8 µL) then immediately cooled on ice (to stop the reaction). 10 µL of 2x reaction mix and 2 µL of AMV reverse transcriptase RT (NEB, Hertfordshire, UK) was added to make the total volume 20 µL. The 20 µL mixture was incubated at 25°C for 5 minutes, and 42°C for 60 minutes, for maximum yield and length, and then further heated to 80°C for 5 minutes to stop the reaction. Samples were diluted to 50 µL by adding 30 µL of RNase free water, and stored at -20°C.

2.4.1.6 Polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify specific sequences of cDNA by million folds, using sequence specific oligonucleotides, heat stable DNA polymerase, and thermal cycling. PCR amplifies DNA exponentially; doubling the number of target molecules with each amplification cycle.

Thermal cycling is based on three major steps: Denaturation, high temperatures break the hydrogen bonds between complementary DNA strands to form single stranded DNA (the highest temperature the DNA can withstand is 95°C); Annealing, depending on the calculated melting temperature (Tm) of the primers (usually 5°C below Tm), short complementary sequences attach to the target DNA template; Elongation of the annealed primer by Taq polymerase to form a new strand of DNA, primer extension occurs at a rate of up to 100 bases per second at the optimum temperature (70-72°C).

2.4.1.6.1 Reverse transcriptase quantitative polymerase chain reaction

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) is used to simultaneously detect a specific DNA sequence in a sample, and determine the sequences copy number relative to a standard. SYBR green (a fluorescent dye) binds to double stranded DNA molecules non-specifically, emitting a fluorescent signal which increases with increasing cycles due to exponential increase in PCR product.

The PCR reaction per sample constituted: 5 µL of iTaq SYBR Green supermix (Bio-Rad, Hertfordshire, UK); 0.25 µL forward (F) and reverse (R) primers (*LGR5* was the target gene and GAPDH was the reference gene) *LGR5* forward (5`-CCTGCTTGACTTTGAGGAAGACC), *LGR5* reverse (5`-CCAGCCATCAAGCAGGTGTTCA), GAPDH forward (5`-AATCCCATCACCATCTTCCA) and GAPDH reverse (5`-TGGACTCCACGACGTACTCA); 1 µL of cDNA; and nuclease free water, up to a final volume of 10 µl. The reaction was set following the conditions optimised for the target at 40 cycles. All samples and negative controls were amplified in triplicate, and the mean value was used for further analysis.

A standard curve was generated so that the efficiency and reproducibility of the qPCR could be assessed. Ishikawa cells (ISK) were used to produce a dilution series of cDNA, spanning the concentration range for the experimental samples amplified. Results were plotted with input nucleic acid quantity on the x-axis, and cycle threshold (Ct) on the y-axis. The reaction efficiency had to be in the accepted range of 90-110%, and this was

determined by the slope of the curve. The R2 value of the standard curve was accepted at ≥0.996, confirming reproducibility of the experiment.

2.4.1.6.2 Gene expression analysis

Gene expression analysis was performed using Bio-Rad CFX Manager software (Hertfordshire, UK), and relative quantification. The expression level of a gene of interest is analysed for up- or down- regulation, looking at fold changes between the sample and the control.

The ΔΔCt method was used to compare experimental sample results with the calibrator (untreated sample), and the normaliser (reference genes). For the gene of interest, the test sample and the calibrator are adjusted in relation to the mean of the normalisers Ct from the same samples. The resulting ΔΔCt value is incorporated to determine the fold difference in the expression.

Fold differences = $2^{-\Delta\Delta Ct}$

ΔC t sample- ΔC t calibrator = ΔΔCt

C t target- C t reference= ΔC t sample

C t target – C t reference = ΔC t calibrator

2.5 *In situ* hybridisation

In situ hybridisation (ISH) allowed for the localisation of a specific segment of nucleic acid within a histological specimen. If nucleic acids are preserved adequately within a specimen, they can be detected through the application of a complementary strand of nucleic acid, to which a reporter molecule is attached. The RNAscope 2.5 High Definition Brown assay was used (Advanced Cell Diagnostics, Hayward, CA) to locate the *LGR5* mRNA in FFPE specimens.

RNA free slides were firstly baked for 1 hour 30 minutes at 60°C to encourage adherence of the tissue to the slide. All containers/surfaces/implements used for the experiment (including the de-paraffinisation steps) were washed in RNASEZAP™, and rinsed with distilled

RNAse free water to eliminate any external RNAs, keeping the appropriate signal as strong as possible. Dewaxing consisted of submerging the samples in fresh xylene (10 minutes) twice, followed by fresh 100% ethanol (2 minutes) twice. Slides were allowed to air dry at RT for 5 minutes (during this time the oven was set to 40°C, and the humidified chamber was assembled following RNASEZAP™ treatment of all components). 1-2 drops of pretreat 1 (to cover the entire section) were added to each sample, and these were incubated for 10 minutes at RT. After washing in distilled water twice, slides were submerged into boiling pretreat 2 (20 mL of pretreat 2 and 180 mL of RNase free water microwaved until gently boiling) for 15 minutes. The slides were washed in distilled water twice and placed in fresh 100% ethanol before air drying at RT. A hydrophobic barrier was drawn around each section and left to completely dry, before 1-2 drops of pretreat 3 were added to each sample, and the slides were placed into the hybridisation chamber in the oven for 30 minutes (during this time the *LGR5* negative and positive probes were placed into an incubator at 40°C to dissolve any precipitation prior to use). Slides were washed in distilled water twice, before being placed back into the hybridisation chamber and adding 2 drops of the appropriate probe (*LGR5* (NM_003667.2, region 560-1589 catalogue number 311021) *POLR2A* (positive control probe) (NM_000937.4, region 2514-3433 catalogue number 310451) and dapB (negative control probe) (EF191515, region 414-862, catalogue number 310043) to the pre labelled sections. The hybridisation chamber was placed in the oven at 40°C degrees for 2 hours (during this time AMPs 1-6 were taken out of the fridge to acclimatise to RT, and 1 L of wash buffer was made (980 mL water and 20 mL wash buffer, warm 50x wash buffer to 40°C degrees for 20 minutes before making, to remove any precipitation)). Following incubation the slides were placed in 1x wash buffer for 2 minutes (twice), and then 2 drops of AMP 1 were added to each section. The slides were placed in the hybridisation chamber in the oven for 30 minutes and the wash step repeated, before 2 drops of AMP 2 are added. Slides were then placed in the hybridisation chamber in the oven for 15 minutes. The wash step was repeated following each AMP application; AMPs 3 and 5 were left for 30 minutes, and AMPs 4 and 6 were left for 15 minutes. Following AMP 4 the hybridisation oven was no longer utilised, and

all steps were performed at RT. DAB substrate was used following the 6 AMP steps in order to detect the signal (equal volumes of Brown A and Brown B were mixed together in a microcentrifuge tube). The slides were incubated with the DAB substrate for 20 minutes at RT before being washed in distilled water twice and counterstained with 50% hematoxylin for 30 seconds. The slides were again washed in distilled water before being submerged in 0.02% ammonia water (33 µL of 30% ammonia and 50 mL water) (Sigma-Aldrich, Dorset, UK) for 30 seconds. After washing in distilled water the slides were dehydrated through 70% ethanol (2 minutes), 100% ethanol (2 minutes twice), and xylene (5 minutes), before being mounted using 1-2 drops of Consul-Mount (Thermo Scientific, Runcorn, UK).

2.5.1 Quantification of *In situ* hybridisation data

LGR5 expression was quantified according to the five-grade scoring system recommended by the manufacturer and previously described (Baker, Graham et al. 2015) (0 = No staining or less than 1 dot to every 10 cells (40× magnification), 1 = 1–3 dots/cell (visible at 20–40× magnification), 2 = 4–10 dots/cell, very few dot clusters (visible at 20–40× magnification), 3 = > 10 dots/cell, less than 10% positive cells have dot clusters (visible at 20× magnification), 4 = > 10 dots/cell, more than 10% positive cells have dot clusters (visible at 20× magnification)).

2.6 Immunohistochemistry

The protein expression of numerous immunohistochemistry (IHC) antibodies were investigated using IHC.

2.6.1 IHC protocol

Tissue sections were baked for one hour at 60°C, prior to commencing the protocol, to encourage adherence of the tissue sections to the slide. The sections were deparaffinised in xylene, and rehydrated through graded alcohol. To enhance immunostaining, antigen retrieval, which involved heating the sections in a pressure cooker for an appropriate time with the appropriate optimised buffer (Table 2.2), was performed. Following antigen

retrieval the sections were incubated in 0.3% hydrogen peroxide/TBS (Sigma-Aldrich, Dorset, UK) for 10 minutes (to block endogenous peroxidase). After blocking unspecific binding with horse serum for 20 minutes (if necessary, see Table 2.2), the primary antibody was added and the sections were incubated in a humidified chamber for the appropriate length of time at the appropriate temperature (Table 2.2). One drop of Vector ImmPRESS labelled polymer-HRP matching the primary antibody (Vector Laboratories, Peterborough, UK) (Table 2.2) was then added to each sample, and they were incubated for 30 minutes, followed by incubation with DAB-substrate for 10 minutes, and immersion in distilled water.

Primary Antibody	Type	Clone	Supplier	Antigen Retrieval	Dilution	Incubation	Secondary
SSEA-1	Monoclonal	MC-480	Biolegend	Tris 3 mins pH=9	1:800	Overnight at 4ºC	Mouse
Pancytokeratin	Monoclonal	C2562	Sigma	Citrate 2 mins pH=6	1:4000	Overnight at 4ºC	Mouse
N Cadherin	Monoclonal	8C11	Abcam	Citrate 2 mins pH=6	1:200	Overnight at 4ºC	Mouse
SOX 9	Polyclonal	SOX 9	R&D	Citrate 4 mins pH=6	1:400	Overnight at 4ºC	Mouse
CK5/6	Monoclonal	D5/16B4	Merck	Tris 3 mins pH=9	1:250	Overnight at 4ºC	Mouse
MUC 1	Monoclonal	BC-2	Serotec	Citrate 2 mins pH=6	1:400	Overnight at 4ºC	Mouse
BCAM	Monoclonal	EPR4164	Abcam	Citrate 2 mins pH=6	1:100	Overnight at 4ºC	Rabbit
CCO	Monoclonal	1D6E1A8	Invitrogen	Citrate 2 mins pH=6	1:1000	30mins room temperature	Mouse
Ki67	Monoclonal	Ki67	Dako	Citrate 4 mins pH=6	1:200	Overnight at 4ºC	Mouse
LGR5-N	Polyclonal	AP2745a	Abgent	Citrate 2 mins pH=6	1:100	Overnight at 4ºC	Rabbit
LGR5-C	Polyclonal	AP2745f	Abgent	Citrate 2 mins pH=6	1:100	Overnight at 4ºC	Rabbit

Table 2.2 – IHC antibodies and conditions of use.

In order to hold the reaction and remove any unbound material (that could potentially interfere with subsequent steps), sections were rinsed in Tris-buffered saline (TBS) at the end of each step.

Lastly, the sections were counterstained with Gill haematoxylin (Thermo Scientific, Runcorn, UK) and mounted in Consul-Mount (Thermo Scientific, Runcorn, UK), after being dehydrated through graded alcohol.

To assess the specificity of the primary antibody, a negative control was included in each run. For this, the primary antibody was substituted with a matched type, and concentration of immunoglobulin. An internal positive control was also included in each run to identify any staining variation.

All slides were scanned using an Aperio CS2 scanner (http://www.leicabiosystems.com/digital-pathology/aperio-digital-pathology-slide-scanners/products/aperio-cs2/. Leica, Wetziar, Germany) and analysed using spectrum, ScanScope® (Leica, Wetziar, Germany).

2.6.2 Analysis of IHC data

Entire sections were evaluated at ×400 magnification for analysis. The proportion of positive staining was used to determine the proliferation index (Ki67); the scanned sections were analysed, and positive staining of any intensity was estimated as a percent. Quick score was used to assess SOX9 and SSEA-1 immunostaining. The whole section was analysed and the proportion of positive cells (0=no staining, 1=25%, 2=50%, 3=75% and 4=100%) was multiplied by the staining intensity (0=no staining, 1=weak, 2=moderate and 3=strong), giving a final score between 0 and 12. All slides were scored by two independent observers who were blinded to the sample identity.

All other IHC protein expression was analysed descriptively with no formal statistical analysis.

2.6.3 Dual staining with *In situ* hybridisation and Immunofluorescence

After performing the aforementioned ISH protocol, slides were washed in PBS, and blocked with horse serum for 30 minutes at RT. SSEA-1 (1:100 Biolegend, San Diego, USA) was added to the samples, and they were left overnight at 4°C in a humidified chamber, before being washed and incubated with the secondary antibody (Alexa FluorR 488 (anti-mouse) Thermo Fisher, Cheshire, UK) for 30 minutes at RT in the dark. Slides were mounted in Vectashield with DAPI (Vector laboratories, Peterborough, UK).

Immunofluorescence (IF) was visualised on a Nikon Eclipse 50i microscope (Nikon, Surrey, UK), using NIS Elements F (Nikon, Surrey, UK) for image capture.

2.7 Explant culture

The endometrial explant cultures were kindly prepared by Dr Sofia Makrydima at our laboratory. Freshly collected endometrial biopsies were firstly washed several times in Dulbecco`s modified Eagle`s medium (DMEM)/F12 (Phenol red free, Life technologies, Paisley, UK) to remove blood. The tissue was cut into 1-2 mm^3 pieces and placed into 24 well plates with medium [DMEM/F12, 5% charcoal-stripped fetal bovine serum (FBS, Sigma-Aldrich, Dorset, UK), Primocin (Source Bioscience, Nottingham, UK)] containing 1 µM medroxyprogesterone acetate (MPA) (Sigma-Aldrich, Dorset, UK), or ethanol (vehicle control), for 24 hours (as previously described) (Valentijn, Saretzki et al. 2015). Harvested tissue, after treatment, was washed with PBS, immersed in RNAlater, and frozen at -80°C for qRT-PCR (Valentijn, Saretzki et al. 2015).

2.8 Three dimensional epithelial cell cultures in Matrigel

The epithelial cells grown in three dimensional (3D) culture, used for assessing *LGR5* expression, were kindly provided by Mr Anthony Valentijn at our laboratory; and the methodology was as previously described (Valentijn, Palial et al. 2013). Short-term cultured (16–36 hours post-plating) epithelial cells, from predominantly proliferative phase samples, were trypsinized and re-suspended to single-cell suspensions at 100,000 cells/200 mL in undiluted Matrigel (BD Biosciences, Oxford, UK), and diluted serially 2-fold up to approximately 3000 cells/100 mL; 50 mL of the resulting mixture was plated in duplicate in 24-well tissue culture plates. After allowing the Matrigel to set at 37°C for 15–20 minutes, DMEM/F12 medium supplemented with insulin-transferrin-selenite (Invitrogen, Paisley, UK), and 50 ng/mL Epidermal growth factor (EGF) (Sigma-Aldrich, Dorset, UK) was added. The medium was

replaced every 3 days, and cultures were monitored over 14 days. Short-term cultured epithelial cells were sorted into SSEA-1^{+} and SSEA1^{-} using magnetic activated cell sorting (MACS), the 2 fractions were embedded in Matrigel as described above. For ISH, the 3D cultures were fixed in 10% neutral-buffered formalin (NBF) (Sigma-Aldrich, Dorset, UK) for 30 minutes, harvested into 1% agarose in PBS, and placed in NBF overnight at 48°C then processed to paraffin wax for sectioning (see RNA free sectioning above).

2.9 **Histochemistry**

Slides were baked for one hour at 60°C, prior to commencing the protocol, to encourage adherence of the tissue to the slide. The sections were deparaffinised in xylene and rehydrated through graded alcohol. Slides were immersed in Gills 2 Haematoxylin (Thermo Scientific, Runcorn, UK) for 4 minutes, before being rinsed in tap water, dipped in acid alcohol, and returned to tap water for 5 minutes. The slides were then placed in 70% ethanol, followed by 95% ethanol, for one minute each, before counterstaining with Eosin Y (Thermo Fisher Scientific, Runcorn, UK) for 4 minutes. Subsequently, they were rinsed in water before dehydrating, clearing, and mounting with Consul-Mount (Thermo Fisher Scientific, Runcorn, UK).

2.10 **Model generation**

100 consecutively cut and stained sections of full thickness endometrium were scanned using an Aperio ScanScope slide scanner (Aperio Technologies, Vista, CA, USA) at x400 magnification, creating virtual slides. The slides were sent to the Leeds Institute of Cancer and Pathology where they underwent the process of `registration,` aligning in stacks, so that the two dimensional (2D) features were aligned to form smooth 3D topographies. This registration resulted in a stack of images which had been aligned, thus could be rendered in 3D as a 3D ‘volume’. This ‘volume’ was a 3D image, which was analogous to a cube of glass containing the stained tissue. The virtual slides were registered using Slice Registration Application

(SliceRegApp) program, [version 11.1.1 (64 bit OpenMP build), University of Leeds, Leeds, UK]. For this, a reference image was selected in the middle of the image stack, and was used to align subsequent images proceeding out from the centre, aligning all images to their neighbours. This aligned each virtual slide to adjacent slides within the dataset, before the images were uploaded into FreeD16 240 software program (Andrey and Maurin 2005) for 3D reconstruction. The method of which was as follows; serial images (TIFF file format) were imported into FreeD software v 1.10 image stack files. Endometrial gland boundaries, in a specific area on the individual sections of full thickness endometrium, were drawn manually in each 2D serial image, and connected along the third dimension, between adjacent slides, producing 3D models.

The different anatomical areas of the full thickness endometrial samples (the basalis and the functionalis) were examined in greater detail, relating to the architecture of the glands, and the gland interactions.

2.11 **Enzyme histochemistry**

A dual histochemical assay was used to determine the cytochrome C oxidase (CCO) status of the glands. In order to identify mutations in the CCO enzyme, dual enzyme histochemistry with succinate dehydrogenase (SDH) was used (the blue SDH staining highlights where the wild type brown staining is absent). Frozen sections were thawed for 1 hour at RT in a moist chamber to prevent drying out and contamination (these samples were subsequently used for laser capture micro dissection (LCM) and DNA extraction). The CCO (500 µm cytochrome C Sigma-Aldrich, Dorset, UK) and DAB (light sensitive) (5 mM DAB in phosphate buffer pH 7.0 Sigma-Aldrich, Dorset, UK) frozen aliquots were initially thawed at 37°C (one pre mixed tube was adequate to do 20 slides), and mixed together with a match head of catalase (20 µg /mL Sigma-Aldrich, Dorset, UK), before vortexing and filtering with a 2 mL syringe. 50-200 µL of the medium was added to each sample (to cover the tissue), before the slides were incubated at 37°C for 55 minutes. Following incubation, the slides were washed 3 times in PBS, and incubated in succinate dehydrogenase (SDH) (1.875 mM NBT in phosphate

buffer pH 7.0 (Sigma-Aldrich, Dorset, UK), 1.3 M sodium succinate in phosphate buffer pH 7.0 (Sigma-Aldrich, Dorset, UK), 2 mM PMS in phosphate buffer pH 7.0 (Sigma-Aldrich, Dorset, UK), 100 mM sodium azide in phosphate buffer pH 7.0)Sigma-Aldrich, Dorset, UK)) at 37°C for 1 hour 20 minutes. The slides were, again, washed in PBS 3 times, before dehydrating in graded ethanol, and air drying for 1 hour prior to LCM. When the slides were for normal tissue processing, they were cleared with `histo clear` (National Diagnostics, Atlanta, Georgia, USA), and mounted in DPX (BDH Laboratory Supplies, Poole, United Kingdom).

2.12 **Laser Capture Micro dissection**

All equipment to be used with the LCM underwent 10 minutes of ultraviolet (UV) treatment in the hood (LCM tubes, tube holder, pipette tips and ATL buffer), in order to remove as much contaminating DNA as possible pre micro dissection, and DNA extraction of the samples.

Single endometrial epithelial cells of interest (mutated and wild type), and stroma for control, were cut into sterile 0.5 mL AdhesiveCap PCR tubes (Carl Zeiss, Oberkochen, Germany) using a PALM Microbeam laser capture system (Carl Zeiss, Oberkochen, Germany). Following LCM, 15 µL of ATL buffer (QIAamp DNA micro kit, Qiagen, Manchester, UK) was added to each sample, to commence cell lysis, and the samples were stored at -20°C overnight, prior to DNA extraction.

2.13 **Isolation of genomic DNA from LCM tissue**

The Qiagen QIAamp DNA micro kit (Qiagen, Manchester, UK) was utilised to extract the DNA from the single epithelial cells.10 µL of proteinase K was added to each sample of epithelial cell and ATL mixture, and the samples were mixed by pulse vortexing for 15 seconds. This was followed by a 3 hour incubation at 56°C (with occasional agitation), before 25 µL of buffer ATL, and 50 µL of buffer AL were added, and the sample was pulse vortexed again for 15 seconds. To ensure efficient cell lysis, the sample and buffer AL were thoroughly mixed to yield a homogenous solution. 50 µL of 100%

ethanol was added to each sample before they were pulse vortexed for 15 seconds, and incubated at RT for 5 minutes. Samples were briefly centrifuged (to remove any residual drops from inside the lid), and carefully transferred to the QIAamp MinElute column (QIAamp DNA micro kit, Qiagen, Manchester, UK) (in a 2 mL collection tube), without wetting the rim. The samples were centrifuged at 6000g for 1 minute before discarding the flow through, and collection tube. This was repeated after adding 500 µL of buffer AW1, and 500 µL of buffer AW2, before the samples were centrifuged at full speed (20,000g) for 3 minutes to dry the membrane completely (this step is necessary as ethanol carryover into the eluate may interfere with downstream applications). The QIAamp MinElute columns were placed in clean 1.5 mL microcentrifuge tubes, and 20 µL of distilled water was added to the centre of the membrane. The samples were then incubated at RT for 5 minutes, spun at maximum for 1 minute, before a further 20 µL of water was added, and the samples were incubated at RT for 5 minutes and spun on maximum for a further minute. The DNA, now in the collection tube was stored at -20°C prior to PCR.

2.14 **Sequencing of mtDNA from individual endometrial epithelia cells**

The entire sequence of the mitochondrial genome from microdissected individual epithelial cells was determined using the single-cell lysate as the DNA template, and a two-stage amplification protocol.

The primary PCR reactions involved amplification of the mitochondrial genome in 9 fragments of approximately 2 kb, using a series of overlapping primer pairs. These initial large PCR reaction products decrease the risk of amplifying pseudogenes when extracting DNA from small quantities of tissue. All PCR amplifications were performed in a 50 µL volume, containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% wt/vol gelatin), 0.2 mM dNTPs, 0.6 µM primers, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems), and 1 µL single-cell lysate. PCR conditions were 94°C for 12 minutes, and 38 cycles of 94°C for 1 minute;

60°C for 1 minute, and 72°C for 2 minutes. The final extension was for 8 minutes.

The secondary PCR reactions involved amplification of the primary PCR products with 28 primer pairs, specifically to generate overlapping fragments of between 600–700 base pairs that span the entire sequence of the human mitochondrial genome. To facilitate the direct sequencing of PCR-amplified products, all primer pairs were tagged with M13 sequence, and designed to anneal optimally at 58°C. All reactions proceeded for 30 cycles, and used 2 µL of primary reaction product as the DNA template.

2.15 **Agarose Gel**

Following PCR, before sequencing, a gel electrophoresis was used to confirm that PCR products were present in the sample. Agarose 1.5 g (Bioline, Essex, UK) and 100 mL of TAE buffer (4.9 L water and 100 mL TAE 50x tris acetate EDTA 10x solution (Severn Biotech, Worcestershire, UK)) were heated in the microwave for 2 minutes, before 5 µL per 100 mL of gel red (nucleic acid gel stain 10,000x in water 0.5 mL (Biotium, Fremonet, USA)) (light sensitive) was added. The hot agarose mixture was poured into the gel tray and allowed to set for 20 minutes. 1.5 µL of loading dye (glycerol, (Thermo Fisher Scientific, Loughborough, UK) and bromophenol blue 6x (Thermo Fisher Scientific, Loughborough, UK), 3 mL glycerol (30%), 25 mg bromophenol blue (0.25%), water to 10 mL) was added to 5 µL of each sample, and the ladder (100bp hyperladder (Bioline, Essex, UK)) was placed into the 1st well of each row. Samples mixed with loading dye were transferred to the gel, and this was run at 135V for 45 minutes, before viewing under UV light (Figure 2.2).

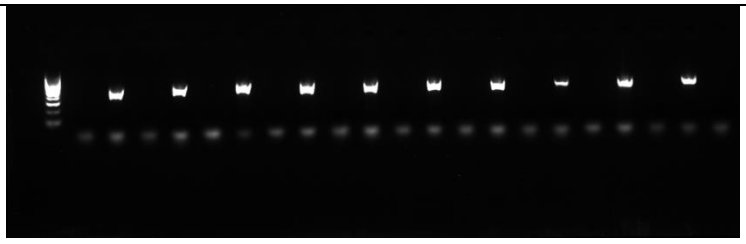


Figure 2.2 – Representative photograph of identified PCR products on a gel.

2.16 **ExoSAP**

If the gel showed that DNA was present, then these samples were treated with ExoSAP (Thermo Fisher Scientific, Loughborough, UK); an enzyme that removes single strand DNA and unused dNTPs, to leave just double stranded DNA. 5 µL of DNA and 2 µL of ExoSAP (kept on ice as it is an enzyme) were added to each well, and the plate underwent the ExoSAP programme in the thermocycler (37°C for 15 minutes, 80°C for 15 minutes, followed by storing at 12°C).

2.17 **Big Dye Terminator reaction**

The Big Dye Terminator (BDT) reaction is necessary to incorporate fluorescent labels into the reaction prior to sequencing (it contains a mixture of fluorescent and non-fluorescent dNTPS). When the PCR incorporates a fluorescent dNTP, the extension of the strand stops leaving a mixture of products of different lengths ending in a fluorescent dye (different colours for Guanine (G), Thymine (T), Adenine (A), Cytosine (C)). The sequencer separates the products by size, and reads the tag on the end.

Following the ExoSAP reaction, 20 µL of water was added to each sample, and 2 µL of this diluted sample was added to the BDT reaction (5 µL BDT, 2.5 µL water, 0.5 µL M13 primer, forward or reverse as universal primer (Sigma primer, F =5`GTAAAACGACGGCCAGT, R=5`CAGGAAACAGCTATGAC)). These samples were run on the PCR ‘mitoseq’ programme (96°C for 1 minute, 96°C 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes, repeat for 24 cycles before holding at 10°C).

2.18 **EDTA-Ethanol cleaning protocol**

Following the BDT reactions, prior to sequencing, the samples were cleaned to remove unincorporated primers. This was done with the EDTA-ethanol cleaning protocol. 10 µL of the sequencing product from the BDT was added to a plate compatible with the sequencer (barcoded). 2.5 µL of 125 mM

EDTA (Thermo Fisher Scientific, Loughborough, UK) was added to each sample, before gentle vortexing, to ensure that the EDTA was not sitting on top of the sample (if this happens, the sample will precipitate on contact with the ethanol). 30 µL of 100% ethanol was added to each sample before vortexing briefly again, and incubating at RT for 20 minutes (minimum) before the plate containing the samples was centrifuged at 4000 rpm for 30 minutes. The supernatant was removed by inverting the plate and spinning at 800 rpm for 20 seconds, before adding 100 µL of 70% ethanol (to wash the pellet), and spinning the plate for 10 minutes at 4000 rpm. The supernatant was removed, as previously described, and the plate was ready to be stored at -20°C to await sequencing; or 10 µL of formamide was added to each sample, and the plate had the denature PCR program run (95°C for 3 minutes), before placing on ice and delivering straight to the sequencer.

2.19 **Sequencing**

The sequencing was completed on a Sanger sequencing reaction, using BigDye 3.1 terminator cycle-sequencing chemistries (Applied Biosystems, Thermo Fisher Scientific, Loughborough, UK) on an ABI 3730XL automated DNA sequencer, by the staff at Barts cancer Institute.

The sequences obtained were analysed using 4Peaks software (www.mekentosj.com), together with Clustal W2 software (EMBL-EBI), and compared to the revised Cambridge reference sequence (Andrews, Kubacka et al. 1999) with sequences from stromal controls and CCO normal specimens to identify polymorphisms and somatic mutations from the CCO-deficient sequences. Individual mutations were confirmed, in all instances, by repeating the first and second-round PCRs, and resequencing the products.

2.20 **Statistical analyses**

Statistical differences between groups were calculated by non-parametric tests, using graph pad prism (GraphPad Software, California, USA). Descriptive values were presented as median and range. The correlations

between immuno-expression scores were examined with Spearman Rank test. P <0.05 was considered significant.

Chapter 3. LGR5 as a stem cell marker in the human endometrium

3.1 Introduction

The human endometrium is a highly regenerative tissue which undergoes over 400 cycles of menstrual shedding and re-growth in a woman's life time. It is composed of two functionally distinct layers: the superficial functionalis, and the deeper basalis.

The functionalis layer is completely shed with menstruation, and fully regenerated within 2 weeks, up to a thickness of 16 mm (Fleischer 1999). This impressive regeneration implies that a stem cell population may reside within the endometrial glands. The location of stem/progenitor cells of the endometrium is postulated to be within the basalis layer (which remains after the menstrual shedding of the functionalis layer) (Prianishnikov 1978, Gargett, Schwab et al. 2016).

Of the two main endometrial specific cell types, the mesenchymal stem cells (MSC) that give rise to stromal cells are well described and studied (Gargett, Schwab et al. 2016). However, the evidence for an endometrial epithelial stem cell population is sparse. Previous work suggests that SSEA-1, nuclear SOX9, and N-cadherin expressing epithelial cell subpopulations have some ability to generate gland-like structures *in vitro* (Valentijn, Palial et al. 2013, Nguyen, Xiao et al. 2017, Turco, Gardner et al. 2017), but, as yet, there are no other epithelial markers with the location, or functional characterisation, suggestive of stem cells specificity described in the endometrium.

Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is a transmembrane receptor (Barker, van Es et al. 2007), which belongs to a family of glycoprotein hormone receptors (Sun, Jackson et al. 2009). LGR5 is a marker of stem cells in various epithelia, such as the intestinal mucosa

(Schuijers and Clevers 2012), gastric mucosa (Barker, Huch et al. 2010), hair follicles (Jaks, Barker et al. 2008), and kidneys (Barker, Rookmaaker et al. 2012). In mammary epithelium, *Lgr5*$^{+}$ cells contribute to both luminal, and basal epithelia (de Visser, Ciampricotti et al. 2012), and are essential to reconstitute mammary glands from single cells (Plaks, Brenot et al. 2013). In the intestine, *LGR5* was shown to be a Wnt target gene, regulating epithelial regeneration with a restricted expression (visualised by *in situ* hybridisation (ISH)) in the intestinal crypt base (Barker, van Es et al. 2007, Schuijers and Clevers 2012). These basal crypt cells were previously proposed to be an adult intestinal stem cell population, but their formal functional confirmation awaited the discovery of a specific marker (Leushacke and Barker 2012). Subsequent work on *Lgr5*$^{+}$ cells from the intestine, using *in vivo* lineage tracing and a heritable-inducible lacZ reporter gene, showed that *Lgr5*$^{+}$ cells are long-lived, multi-potent stem cells (Gerbe, van Es et al. 2011), and a single *Lgr5*$^{+}$ stem cell can form organoids with a gut-like architecture containing all epithelial cell types (Schuijers and Clevers 2012).

LGR5 is expressed in the female reproductive organs. *Lgr5* marks stem/progenitor cells of the rodent ovary, and the oviduct (Flesken-Nikitin, Hwang et al. 2013, Ng, Tan et al. 2014), where it is critical for the maintenance of a functional corpus luteum and, therefore, for successful pregnancy (Sun, Terakawa et al. 2014). In immature, and in ovarian hormone deprived mice, *Lgr5* is highly expressed in the single layer of epithelia lining the uterine cavity, and progesterone treatment down-regulated *Lgr5*, suggesting an ovarian hormonal regulation (Sun, Jackson et al. 2009, Boretto, Cox et al. 2017). However, mice do not menstruate, their oestrous cycle is characterised by complete reabsorption of the endometrial lining and, therefore, their epithelial regeneration pattern is proposed to be distinct from women (Gargett, Schwab et al. 2016). In the human ovary and distal Fallopian tube (fimbriae), *LGR5* expression was confirmed by quantitative reverse transcription PCR (qRT-PCR) (Ng, Tan et al. 2014), with constitutive *LGR5* mRNA expression reported in healthy human endometrial epithelium throughout the menstrual cycle (Krusche, Kroll et al. 2007, Schuijers and Clevers 2012).

The specificity of the available anti-human-LGR5 antibodies is disputed and, in general, the antibody based protein expression data does not correlate with RNA data (Munoz, Stange et al. 2012). Thus, ISH is considered as the gold standard to detect *LGR5* expressing cells in a solid tissue (Munoz, Stange et al. 2012). An antibody-based approach has been previously reported to locate *LGR5* expressing cells in the human endometrium by a single group, in two recent manuscripts. The first manuscript, which was a review article, presented original data of LGR5 expressing cells being in the perivascular region of the stromal and epithelial compartments, with one Figure depicting 4 immunohistochemistry (IHC) pictures. The authors claimed that the staining intensity, and amount of *LGR5,* did not alter across the menstrual cycle, but due to the type of article (review), the basic methodological information required to assess the scientific robustness of the data, such as the information on the antibody used (origin, clone, type of, etc.) or the number/type (full thickness/pipelle, pathological/non pathological etc.) of samples, was not documented. The authors, however, concluded that LGR5 could be a universal stem cell marker, despite not providing any supporting evidence to that end (Gil-Sanchis, Cervello et al. 2013). The same group, subsequently, published a second manuscript, in which they isolated epithelial and stromal LGR5 cells with fluorescence activated cell sorting (FACS), using a polyclonal rabbit antibody (Novus 28904) without confirmed specificity, to further characterise these cells. They did not show any confirmation of high LGR5 expression in the sorted cells with PCR, or other methods. The characterisation work confirmed the sorted cells to be of haemopoietic origin, leading the authors to suggest that LGR5 was not a universal stem cell marker, and the endometrial cells isolated using the LGR5 antibody were recruited from blood to be part of the stem cell niche, proposed at the perivascular microenvironment (Cervello, Gil-Sanchis et al. 2017). Therefore, in the human endometrium, these antibody-based studies need further validation (Gil-Sanchis, Cervello et al. 2013, Cervello, Gil-Sanchis et al. 2017) in order to confirm the exact LGR5 expressing cell population, and to elucidate the function and regulation of the *LGR5* gene in those cells.

The overall aim of the work described in this chapter was to examine if the expression of the proposed universal epithelial stem cell marker (*LGR5)* is suggestive of demarcating a stem cell population in the human endometrium. We examined the cellular localisation of *LGR5* in all epithelial compartments of the human endometrium by ISH. As the human Fallopian tube shares the same embryological origin, and exists as a continuum with the endometrium, we compared the expression of *LGR5* in the epithelial mucosa of the endometrium, with that of the fimbrial end of the Fallopian tube (due to its known stem cell enrichment (Auersperg 2013) and *Lgr5* expression (Ng, Tan et al. 2014)). The hormone regulation of *LGR5* in the endometrium was also studied *in vitro* and *in vivo*.

3.1.1 The Research Questions that were addressed

1. Are there spatio-temporal differences in *LGR5* expression, in pre-, and post-menopausal (PM) full thickness endometrium, and Fallopian tube?
2. Does endometrial *LGR5* expression correlate with epithelial proliferative activity?
3. Does endometrial *LGR5* expression correlate with the previously known epithelia progenitor markers SSEA-1 and SOX9?
4. Does progesterone alter the expression of *LGR5 in vitro* or *in vivo*?
5. Does LGR5 expression alteration, seen within the progesterone treated human endometrial samples, correlate with cell proliferation?
6. Do isolated SSEA-1 enriched epithelial cells grown in 3D culture, to simulate epithelial glandular regeneration, contain *LGR5* expressing cells?

3.2 Methods

3.2.1 Patient population

Human endometrium and tubal fimbriae was obtained from 57 women undergoing benign gynaecological surgery with no endometrial pathology, at the Liverpool Women's Hospital (Table 3.1) from 2009-2017. The cycle

phase of the endometrium was assigned according to the last menstrual period (LMP), and histological criteria (Noyes, Hertig et al. 1975, Dallenbach-Hellweg 2012). Endometrium and the distal (fimbrial) end of the Fallopian tube samples were divided in to two pieces: one was fixed (⩾24 hours in 4% (v/v) buffered formalin) and paraffin-embedded for ISH and IHC staining; the other piece was immediately placed in to RNA*later* (Sigma, Dorset, UK) for RNA extraction and qRT-PCR. A further 13 endometrial samples were collected in reduced serum (1%) Dulbecco's modified Eagle's medium (DMEM)/F12 media, for short-term explant culture or cell sorting, and three dimensional (3D) culture. ISH and IHC staining for all antibodies was analysed with specific reference to the three different endometrial epithelial compartments in full-thickness endometrial tissue sections: the luminal epithelium (LE) (the single layer of cells that forms the luminal surface, or lining of the uterine cavity); the functionalis (glands in the upper two-thirds of the endometrium below the LE, surrounded by sparse stroma); and the basalis (glands in the lower one-third of the endometrium, adjacent to the endo-myometrial junction, surrounded by densely packed stroma). Sequential sections were stained with pancytokeratin to confirm the assignment of the epithelial compartment.

Sample no.	Stage of cycle/treatment	Age	BMI	Smoker	Parity
1	Proliferative	37	22.8	Yes	2
2	Proliferative	31	43.9	No	1
3	Proliferative	57	22	No	3
4	Proliferative	42	25.6	Yes	2
5	Proliferative	39	36.9	No	4
6	Proliferative	50	21	No	4
7	Proliferative	32	27.8	Yes	2
8	Proliferative	45	36.4	No	3

9	Proliferative	43	40.5	No	3
10	Proliferative	48	37.3	No	2
11	Proliferative	37	39.2	No	2
12	Proliferative	44	29.6	No	1
13	Proliferative	44	24.5	No	2
14	Secretory	32	26.6	No	2
15	Secretory	45	31.6	No	0
16	Secretory	35	32	No	3
17	Secretory	45	26.1	No	0
18	Secretory	47	22.6	No	0
19	Secretory	44	30.7	Yes	2
20	Secretory	37	21.7	No	4
21	Secretory	41	18.9	Yes	2
22	Secretory	39	22.4	Yes	6
23	Secretory	21	25.9	No	0
24	Secretory	46	25.4	Yes	1
25	Secretory	33	26.1	No	2
26	Secretory	40	33	No	2
27	POP	36	23.9	No	4
28	POP	33	27.4	Yes	3
29	POP	25	20.9	No	0
30	POP	31	25.8	Yes	2
31	POP	37	25.7	No	0

32	POP	43	24.2	Yes	2
33	LNG-IUS	46	26.2	No	3
34	LNG-IUS	45	22.5	No	2
35	LNG-IUS	35	24.4	No	1
36	LNG-IUS	33	32.7	Yes	3
37	LNG-IUS	33	25.9	No	5
38	Explant	26	33.4	Yes	2
39	Explant	44	30.7	Yes	2
40	Explant	27	33.7	No	2
41	Explant	40	33	No	2
42	Explant	43	25.4	No	2
43	Explant	47	24.2	Yes	0
44	Explant	48	28.6	No	2
45	PM	69	24.7	No	4
46	PM	69	26.8	No	3
47	PM	67	32.8	No	4
48	PM	66	24.9	No	3
49	PM	52	39.6	No	2
50	PM	74	35.6	No	3
51	SSEA1 sorted	30	26.7	No	1
52	SSEA1 sorted	27	21.1	No	4
53	SSEA1 sorted	40	27.4	No	
54	SSEA1 sorted	38	26.6	Yes	2

55	SSEA1 sorted	27	23	No	5
56	SSEA1 sorted	39	24.8	No	0
57	SSEA1 sorted	31	18.7	No	0

Table 3.1 - Demographical details of the patients included in the study. (Abbreviations POP - Progesterone only pill, LNG-IUS - levonorgestrel-releasing intrauterine system, PM – postmenopausal).

3.2.2 qRT-PCR

Total RNA from tissue samples was extracted using TRIzol Plus RNA Purification System (Life Technologies, Paisley, UK), and quantified by NanoDrop ND-1000 (Thermo Fisher Scientific, Loughborough, UK). Total RNA was reverse transcribed using AMV First Strand cDNA synthesis kit (New England Bio Labs, Hertfordshire, UK) after DNase treatment (DNase I, New England Bio Labs, Hertfordshire, UK), using the manufacturer's protocol as previously described (Kamal, Bulmer et al. 2016). cDNA was amplified by qPCR using iTaq SYBR Green supermix (Bio-Rad, Hertfordshire, UK), with the Bio-Rad connect (Hertfordshire, UK), and the following primers: LGR5 forward (5`-CCTGCTTGACTTTGAGGAAGACC); LGR5 reverse (5`-CCAGCCATCAAGCAGGTGTTCA); GAPDH forward (5`-AATCCCATCACCATCTTCCA); and GAPDH reverse (5`-TGGACTCCACGACGTACTCA). Relative transcript expression was calculated using the ΔΔCT method and normalised to the reference gene, GAPDH, using Bio-Rad CFX manager (Hertfordshire, UK).

3.2.3 *In situ* hybridisation

ISH for *LGR5* expression was performed as previously described (Baker, Graham et al. 2015) on 4 µm sections using the RNAscope 2.5 High Definition Brown assay, according to the manufacturer's instructions (Advanced Cell Diagnostics, Hayward, CA). Formalin-fixed paraffin-embedded (FFPE) tissue sections were baked at 60°C for 1 hour 30 minutes, followed by de-paraffinization, and incubation with Pretreat 1 buffer, for 10

minutes, at room temperature (RT). These slides were subsequently boiled in Pretreat 2 buffer for 15 minutes, followed by incubation with Pretreat 3 buffer for 15 minutes at 40°C. The sections were then incubated with the relevant probes for 2 hours at 40°C, followed by successive incubations with AMPs 1 to 6 reagents. Staining was visualized with 3,3′-diaminobenzine (DAB) for 20 minutes, then lightly counterstained with Gill's haemotoxylin. RNAscope probes used were *LGR5* (NM_003667.2, region 560-1589 catalogue number 311021), *POLR2A* (positive control probe) (NM_000937.4, region 2514-3433 catalogue number 310451), and dapB (negative control probe) (EF191515, region 414-862, catalogue number 310043) (Figure 3.1).

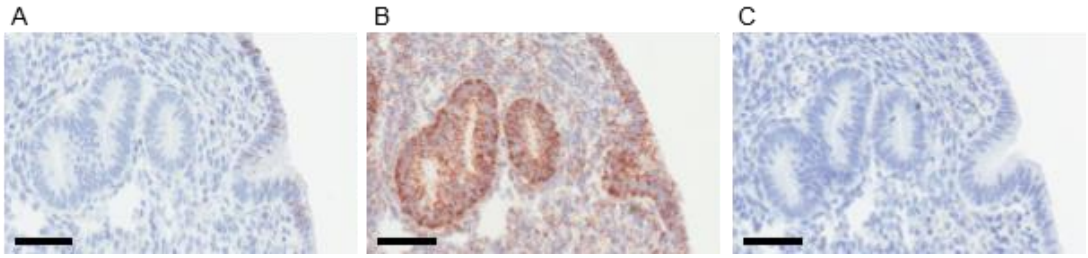


Figure 3.1 - Representative *LGR5* ISH images of secretory endometrium showing (A) *LGR5* Probe (B) Positive control (C) Negative control (All images x400, scale bar = 60µm).

LGR5 expression was quantified according to the five-grade scoring system recommended by the manufacturer previously described (Baker, Graham et al. 2015) (0 = No staining or less than 1 dot to every 10 cells (400× magnification), 1 = 1–3 dots/cell (visible at 200–400× magnification), 2 = 4–10 dots/cell, very few dot clusters (visible at 200–400× magnification), 3 = > 10 dots/cell, less than 10% positive cells have dot clusters (visible at 200× magnification), 4 = > 10 dots/cell, more than 10% positive cells have dot clusters (visible at 200× magnification)).

3.2.4 Immunohistochemistry

After antigen retrieval in citrate, or TRIS buffer (see antibody conditions, Table 3.2), (as described in chapter 2), 4 µm FFPE sections were blocked with 3% hydrogen peroxide for 10 minutes, then immunostained with the

appropriate antibody for the given time, and dilution in Table 3.2. This was followed by incubation for 30 minutes at RT in the appropriate secondary antibody. Detection was with ImmPRESS polymer based system, and visualisation was with ImmPACT DAB (Vector Labarotories, Peterborough, UK), used as per manufacturer's instructions. Sections were counter stained in Gill 2 Haemotoylin (Thermo Scientific, Runcorn, UK), dehydrated, cleared, and mounted in synthetic resin. Matching isotype replaced the primary antibody as a negative control, with internal positive control in each staining run.

Primary Antibody	Type	Clone	Supplier	Antigen Retrieval	Dilution	Incubation	Secondary
SSEA-1	Monoclonal	125601/2	Biolegend	Tris 3 mins	1:800	Overnight at 4ºC	Mouse
Pancytokeratin	Monoclonal	C2562	Sigma	Citrate 2 mins	1:4000	Overnight at 4ºC	Mouse
SOX 9	Polyclonal	Af3075	R&D	Citrate 4 mins	1:400	Overnight at 4ºC	Goat
Ki67	Monoclonal	NCL-Ki67-MM1	Novocastra	Citrate 4 mins	1:200	Overnight at 4ºC	Mouse
LGR5-N	Polyclonal	AP2745a	Abgent	Citrate 2 mins	1:100	Overnight at 4ºC	Rabbit
LGR5-C	Polyclonal	AP2745f	Abgent	Citrate 2 mins	1:100	Overnight at 4ºC	Rabbit

Table 3.2 - Primary antibodies and their immunohistochemistry conditions.

All slides were scanned using an Aperio CS2 scanner (http://www.leicabiosystems.com/digital-pathology/aperio-digital-pathology-slide-scanners/products/aperio-cs2/), and analysed using spectrum, ScanScope®.

3.2.5 Analysis of IHC

Percentage of nuclear Ki67 immuno-positive cells, of any intensity, was evaluated as the Ki67-labelling index (Ki67-LI). The entire section was evaluated at ×400 magnification (minimum 25 fields) (as described previously) (Al Kushi, Lim et al. 2002), and the 3 epithelial compartments

were scored separately. SOX9 and SSEA-1 immunostaining was assessed by multiplying the proportion of positive cells (0=no staining, 1=25%, 2=50%, 3=75% and 4=100%) by the staining intensity (0=no staining, 1=weak, 2=moderate and 3=strong), giving a final score between 0 and 12 (Valentijn, Palial et al. 2013). All slides were scored by two independent observers who were masked to the sample identity.

3.2.6 *In situ* hybridisation with Immunofluorescence

Following on from the ISH protocol, three specimens were used for immunofluorescence (IF) SSEA-1 co localisation. The slides were washed in PBS, rather than mounted, and incubated in horse serum block for 30mins, at RT. SSEA-1 (1:100 Biolegend) was added to the samples, and they were left overnight at 4^{o}C, before being washed and incubated with the secondary antibody, Alexa FluorR 488 (anti-mouse), for 30 minutes at RT, in the dark. Slides were mounted in Vectashield with DAPI (Vector laboratories, Peterborough, UK). IF was visualised on a Nikon Eclipse 50i microscope, using NIS Elements F for image capture.

3.2.7 Explant culture

The endometrial explant cultures were performed, and kindly provided, by Dr Sofia Makrydima at our laboratory. *In vitro*, short-term endometrial explant cultures were prepared from freshly collected endometrial biopsies, which were firstly washed several time in Dulbecco`s modified Eagle`s medium (DMEM)/F12 (Phenol red free, Life technologies, Paisley, UK) to remove blood. The tissue was cut into 1-2mm^3 pieces, and placed into 24 well plates with medium [DMEM/F12, 5% charcoal-stripped fetal bovine serum (FBS, Sigma-Aldrich, Dorset, UK), Primocin (Source Bioscience, Nottingham, UK)], containing 1 µM medroxyprogesterone acetate (MPA) (Sigma-Aldrich, Dorset, UK), or ethanol (vehicle control), for 24 hours as previously described (Valentijn, Saretzki et al. 2015). Harvested tissue, after treatment, was washed with PBS, immersed in RNA*later,* and frozen for qRT-PCR (Valentijn, Saretzki et al. 2015).

3.2.8 3D epithelial cell cultures in Matrigel

The epithelial cells, grown in 3D culture, used for assessing *LGR5* expression, were kindly provided by Mr Anthony Valentijn at our laboratory, and the methodology was as previously described (Valentijn, Palial et al. 2013). Briefly, short-term cultured (16–36 hours post-plating) epithelial cells, from predominantly proliferative phase samples, were trypsinized and re-suspended, to single-cell suspensions at 100 000 cells/200 mL undiluted Matrigel (BD Biosciences, Oxford, UK), and diluted serially 2-fold up to approximately 3000 cells/100 mL; 50 mL of the resulting mixture was plated in duplicate in 24-well tissue culture plates. After allowing the Matrigel to set at 37°C for 15–20 minutes, DMEM/F12 medium, supplemented with insulin-transferrin-selenite (Invitrogen) and 50 ng/mL Epidermal growth factor (EGF) (Sigma-Aldrich), was added. The medium was replaced every 3 days and cultures monitored over 14 days. Short-term cultured epithelial cells were sorted into SSEA-1^{+} and SSEA1^{-}, the 2 fractions were embedded in Matrigel. For ISH, the 3D cultures were fixed in 10% NBF for 30 minutes, harvested into 1% agarose in Phosphate buffered saline (PBS), and placed in NBF overnight at 48°C, then processed to paraffin wax; 4 µm sections were cut.

3.2.9 Statistical methods

All statistical analyses were performed using GraphPad Prism software (Mann Whitney U and one-way ANOVA was used to assess differences between groups). Spearman rank correlation was used to determine the association between pairs of variables. The criterion for significance was $p \leq 0.05$.

3.3 Results

Healthy human pre-menopausal endometrium demonstrated dynamic spatio-temporal regulation of LGR5 expression, with high LGR5 expressing cells in the luminal, and in the basalis epithelium.

Full thickness, whole endometrial tissue samples, containing all endometrial layers and cell types from the oestrogen dominant proliferative phase of the

cycle, showed a trend of higher *LGR5* mRNA expression levels (as measured by qRT-PCR); compared with the samples from the progesterone dominant secretory phase of the menstrual cycle (p=0.5, Figure 3.2A). *LGR5* mRNA levels were significantly higher in the stem cell rich distal Fallopian tubes, than in the corresponding eutopic endometrium (p<0.01, Figure 3.2B). The cell type expressing *LGR5* was identified with ISH, demonstrating that *LGR5* expression was limited to the epithelial compartment in both endometrium and tube. Semi-quantitative scoring of *LGR5* expression revealed that the LE cells expressed significantly higher levels of *LGR5* than all other epithelial compartments in the endometrium (p<0.05, Figures 3.2C and 3.2D), including the endometrial basalis. The reduction in *LGR5* expression in the secretory phase was confirmed with ISH in the LE (p=0.03), and functionalis epithelium (p=0.04) respectively (Figures 2C and 2D).

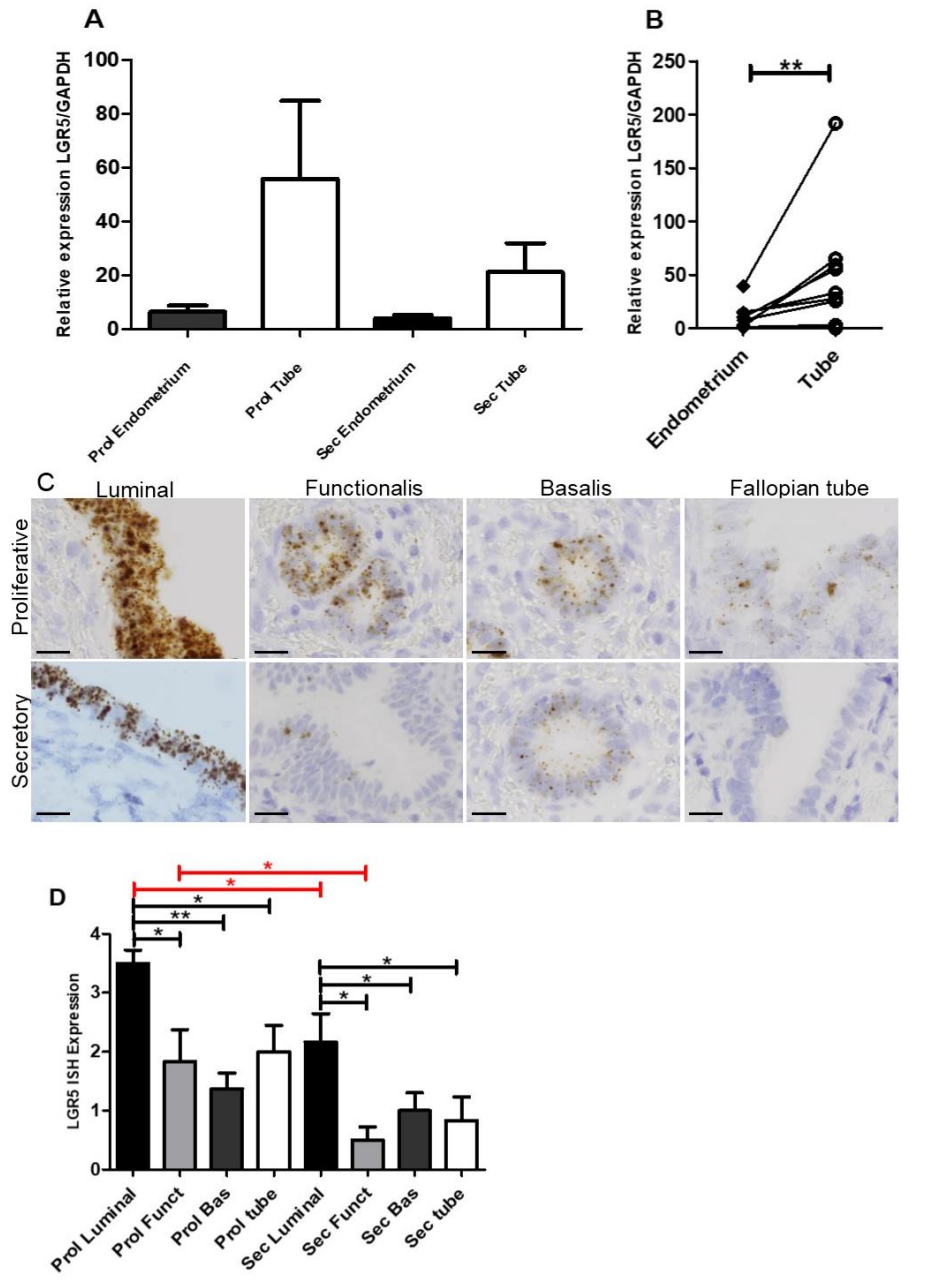


Figure 3.2 - ***LGR5* gene expression appears to decrease in the eutopic endometrium and Fallopian tube in the secretory phase of the menstrual cycle**. (A) The eutopic endometrial samples and matched Fallopian tube express apparently decreased levels of *LGR5* mRNA in the secretory phase of the menstrual cycle when compared with the proliferative phase (n=21). (B) Fallopian tube (at any stage of the cycle) demonstrate significantly higher levels of *LGR5* mRNA than matched eutopic endometrium ($p<0.01$) (n=20). (C) Representative *LGR5* ISH images of Fallopian tube and luminal, functionalis and basalis eutopic endometrium in

the proliferative and secretory stages of the menstrual cycle (All images x1000, scale bar = 20 µm, (n=15). (D) Graphical representation of semi-quantitative scoring of *LGR5* ISH In the proliferative stage of the cycle, the LE demonstrated significantly higher *LGR5* ISH staining scores than the functionalis ($p<0.05$), basalis epithelium ($p<0.01$) and Fallopian tube ($p<0.02$ as well as the LE of the secretory phase ($p<0.03$); the proliferative functionalis had significantly higher *LGR5* scores than the secretory functionalis ($p<0.04$); the secretory LE showed significantly higher *LGR5* scores than the epithelia of secretory functionalis ($p<0.03$), secretory basalis ($p<0.05$) and Fallopian tube ($p<0.02$) (n=7 per group). (Prol=Proliferative, Sec=Secretory, Funct= Functionalis, Bas=Basalis).

LGR5 expression correlated with endometrial epithelial cell proliferation in the functionalis epithelial compartment.

The differences in the cellular proliferative activity in the three endometrial epithelial compartments across the menstrual cycle, were demonstrated by the dynamic changes in the expression of the proliferative marker Ki67 (Figures 3.3A and 3.3B) in sequential tissue sections. Epithelial Ki67-LI was highest in the proliferative phase, with the maximum Ki67-LI observed in the cells of functionalis glands (median 70%, range 10-100%), and the lowest Ki67-LI seen in the basalis epithelium (median 30%, range 0-85%). Ki67-LI was higher in the functionalis and in LE, compared with the basalis glands in all phases of the cycle. In the secretory phase Ki67-LI, in all epithelial compartments, decreased, with the LE compartment demonstrating the highest Ki67-LI (median 1%, range 0-90%); and Ki67-LI was absent in the basalis glands. Ki67-LI and *LGR5* expression levels only correlated significantly ($r=0.74$, $p=0.01$) in the functionalis epithelium. The basalis *LGR5* expression persisted in the secretory phase (Figure 3.2D), while the corresponding Ki67-LI reactivity decreased significantly ($p=0.03$) (Figure 3.3B). The quiescent (absent Ki67-LI) atrophic PM endometrial epithelium also expressed *LGR5* (particularly the LE) (Figure 3.4).

Epithelial proliferation (Ki67-LI) in the Fallopian tube was consistently very low throughout the cycle, contrasting with the dynamic tubal *LGR5* expression pattern (r=0.23, p=0.55).

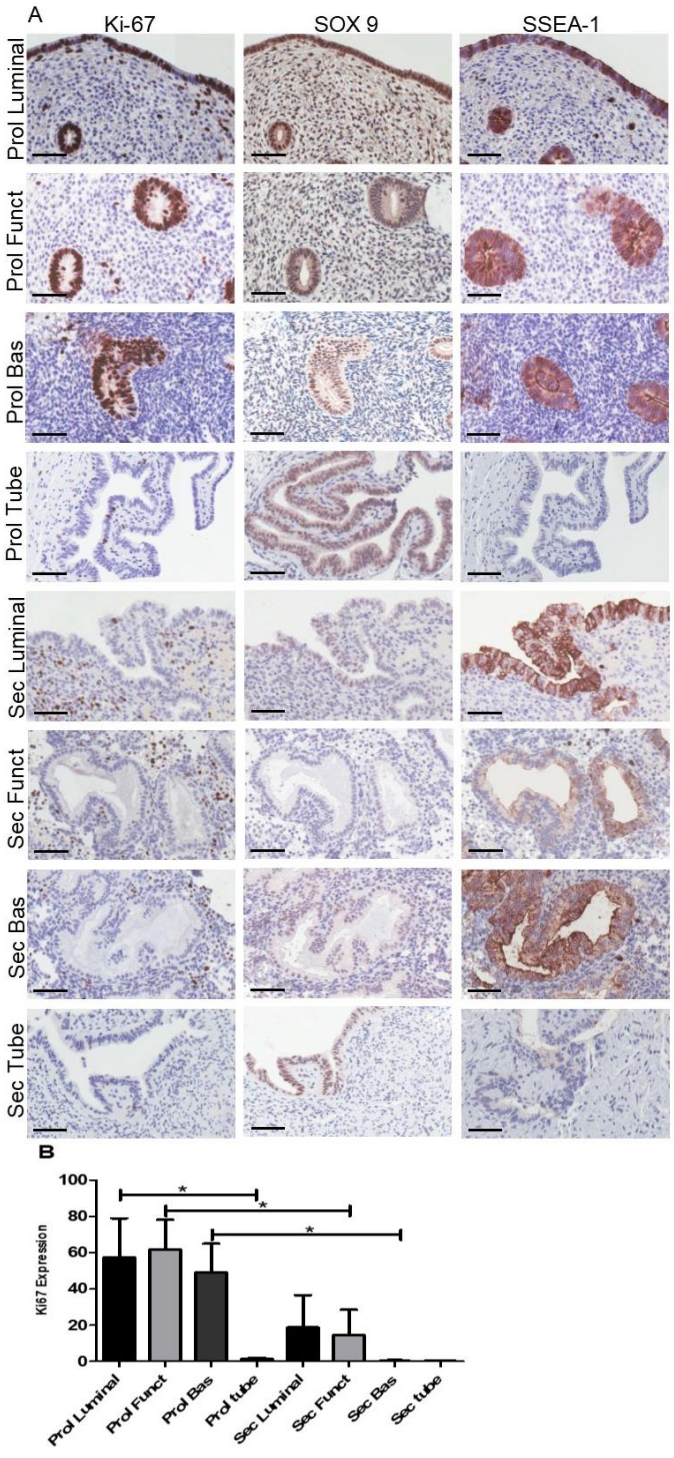


Figure 3.3 - **Ki67-LI correlated with endometrial epithelial cell proliferation only in the epithelial compartment of the functionalis.**

Epithelial *LGR5* expression scores also correlated with the expression of the previously known progenitor markers SOX9 and SSEA-1 in sequential tissue sections across the cycle. (A) Representative images of Ki67, SOX9 and SSEA-1 IHC in luminal, functionalis, basalis epithelial compartments of the eutopic endometrium and Fallopian tubes in the proliferative and secretory stages of the menstrual cycle (all images x400, scale bar 10 µm). (B) Quantification of percentage Ki67-positive cells (Ki-67 LI) throughout the cycle. A minimum of 25 fields of cells were counted at x400 magnification (n=7 per group). The functionalis in the proliferative phase has the highest Ki67-LI and is statistically higher than the functionalis epithelium in the secretory phase ($p<0.05$). Ki67-LI decreased dramatically in the secretory phase of the cycle in all epithelial compartments. (Prol=Proliferative, Sec=Secretory Funct= Functionalis, Bas= Basalis).

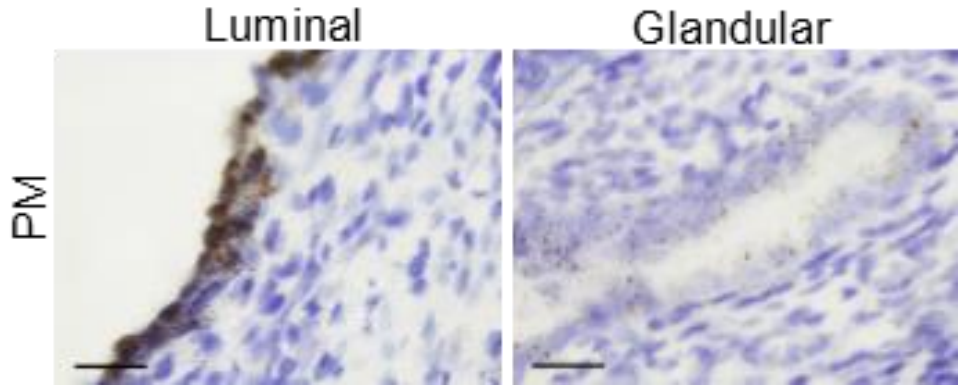


Figure 3.4 - Representative *LGR5* ISH images of PM luminal and glandular epithelium (All images x1000, scale bar = 20 µm).

In the human endometrium, luminal and basalis epithelia share distinct patterns of co-expression of LGR5, and the previously known progenitor markers SSEA-1 and SOX9.

Sequential tissue sections were employed to examine if the cellular location of *LGR5* mRNA (by ISH) was consistent with the expression of, previously described, endometrial basalis progenitor markers SSEA-1 and nuclear SOX9 (by IHC, Figure 3.3A). In general, SOX9 and SSEA-1 expression

followed the same cyclical pattern of expression as *LGR5*: levels decreased in all 3 endometrial epithelial compartments, and also in the tubal epithelium, in the secretory phase, when compared with the samples from the proliferative phase (Figure 3.3A). However, out of all 3 endometrial epithelial compartments, the strongest *LGR5* expression was seen in the LE (Figures 3.2C and 3.2D), whereas the strongest SSEA-1 and SOX9 staining was observed in the basalis glands, agreeing with previous reports (Valentijn, Palial et al. 2013) (Figure 3.3A). It is noteworthy that the luminal staining for both SSEA-1 and SOX9 was consistently high throughout the cycle, even when their expression decreased in the functionalis epithelium in the secretory phase (Figure 3.3A).

In the Fallopian tube, SOX9 staining scores and *LGR5* ISH scores, were high throughout the menstrual cycle, similar to the basalis glands of the endometrium, with only an apparent reduction in the intensity during the secretory phase (Figure 3.2D). In contrast, SSEA-1 scores were very low in the tubal epithelium in all phases of the cycle. The co-expression of SSEA-1 protein and *LGR5* mRNA by ISH was further confirmed with immunofluorescence staining (Figure 3.5).

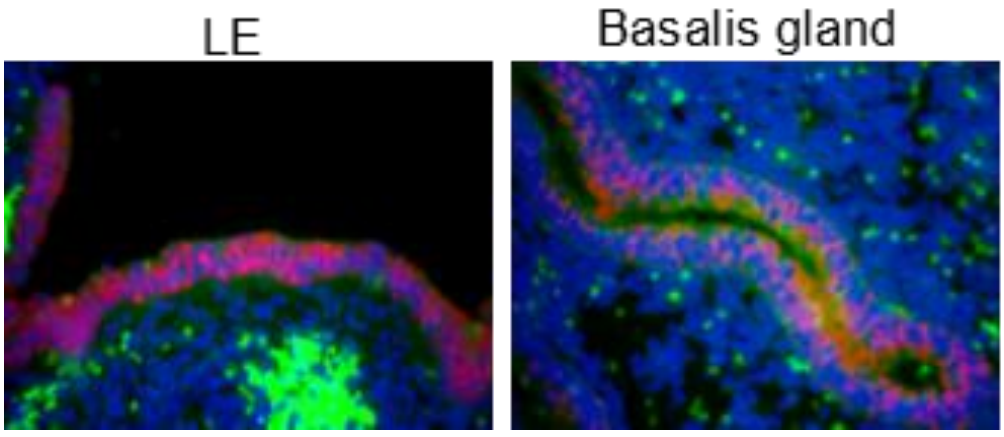


Figure 3.5 – Representative co-localisation *LGR5* ISH probe (red)/anti-human SSEA-1 antibody immunofluorescence (green), images of luminal and basalis epithelium of healthy human full thickness endometrium (All images x400).

Progestogens regulate LGR5 expression *in vitro* and *in vivo*.

The progestogenic regulation of *LGR5* expression was examined by treating endometrial explants *in vitro* with the synthetic progestogen MPA in short-term culture, and MPA treatment decreased *LGR5* levels by 1.5 fold (Figure 3.6A).

The *in vivo* effect of progestogens on the endometrial expression of *LGR5* was tested in endometrial samples from women taking synthetic progestogen treatment (progesterone only pill, 'POP', or levonorgestrel-releasing intrauterine system, 'LNG-IUS') and a significant reduction of *LGR5* mRNA levels were observed with progestogen treatment, compared with the normal eutopic endometrium of women not on hormonal treatments ($p<0.01$, Figure 3.6B). Even with these very low levels, the LE continued to retain higher *LGR5* expression than the glands, following progestogen treatment (Figures 3.6C and 3.6D).

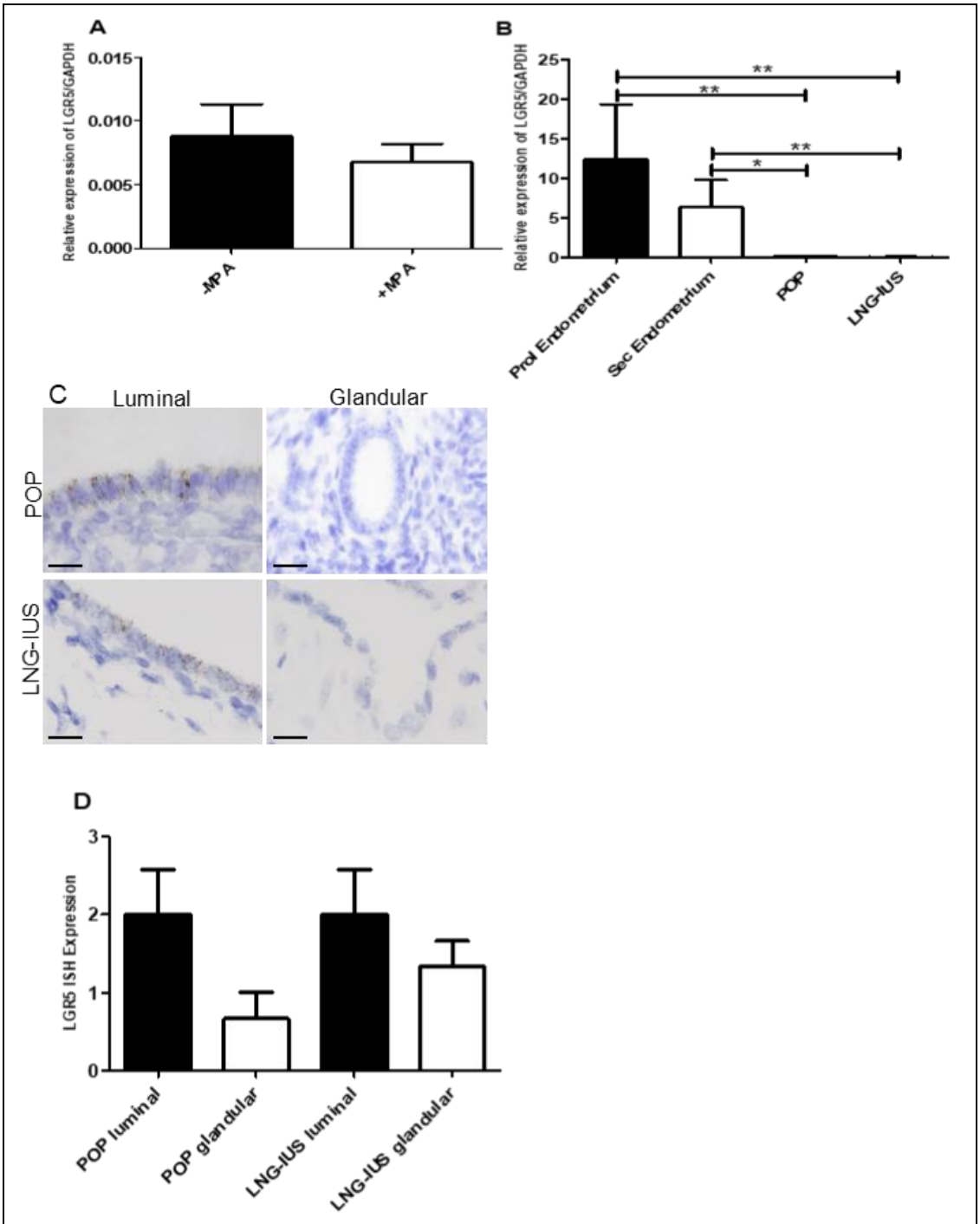


Figure 3.6 - **Progestagens regulate LGR5 expression *in vitro* and *in vivo*.** (A) *LGR5* mRNA expression levels analysed by qRT-PCR. Explants treated with MPA ('+MPA') expressed lower *LGR5* levels relative to GAPDH when compared with the same of vehicle-treated explants ('-MPA') (n=6 per group). (B) *LGR5* mRNA expression by qRT-PCR. Patients taking the oral progesterone only pill (POP) or having the levonorgestrel-releasing intrauterine system (LNG-IUS) have significantly less *LGR5* mRNA

expression relative to GAPDH when compared with normal eutopic proliferative and secretory endometrium ($p<0.01$ and $p<0.04$ respectively). (n = 6 per group). (C) Representative *LGR5* ISH images of POP treated and LNG-IUS treated luminal and glandular eutopic endometrium (All images x1000, scale bar = 20 µm). (D) Graphical representation of semi-quantitative scoring of *LGR5* ISH. The LE has more *LGR5* when compared with the glandular epithelium in the POP and LNG-IUS treated samples (n=6 per group).

LGR5 expression did not correlate with epithelial cell proliferation in progesterone treated human endometrial samples.

The luminal and glandular epithelial Ki67-LI was much higher in the POP samples when compared to the LNG-IUS treated endometrium (POP luminal median 50%, range 40-75%, glands median 70%, range 50-85%, LNG-IUS luminal median 1%, range 0-1%, glands median 2% range 0-2%, Figures 3.7A and 3.7B). Therefore, the Ki67-LI levels in the POP treated samples did not correlate with the levels of *LGR5* expression, whereas, in the atrophic glandular and LE cells of the LNG-IUS samples, there were low levels of both *LGR5* and Ki67-LI.

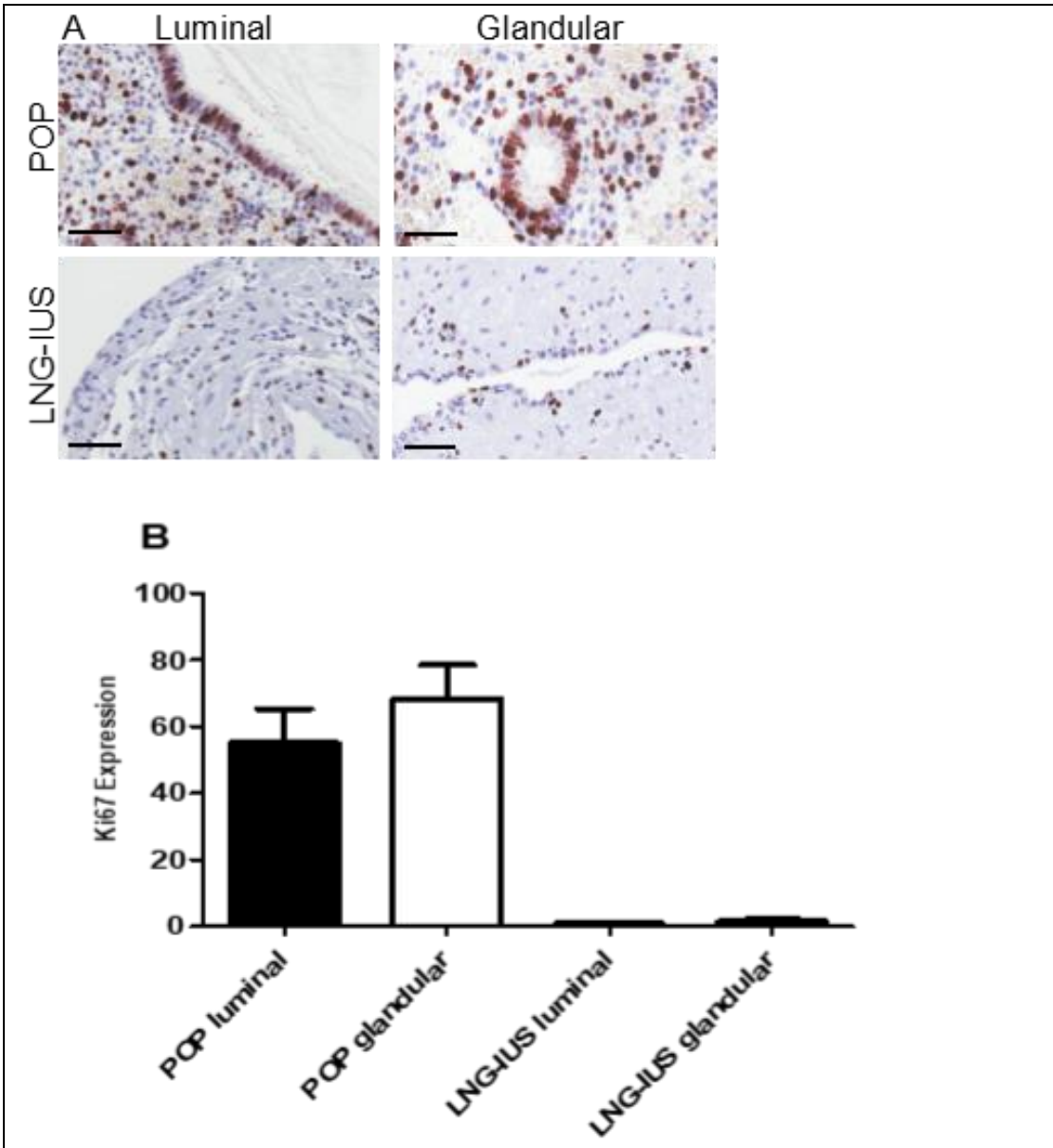


Figure 3.7 - ***LGR5* expression did not correlate with epithelial cell proliferation in progesterone treated human endometrial samples.** (A) Ki67-LI IHC data, in the progesterone only pill (POP) group, the glandular and LE showed high Ki67-LI but the levonorgestrel-releasing intrauterine system (LNG-IUS) group endometrial Ki67-LI was very low. (B) Representative Ki67 images of luminal and glandular eutopic endometrium from patients treated with either POP or LNG-IUS (all images x400, scale bar =10 µm, n=6 per group).

Sorted SSEA1$^+$ epithelial cells grown in 3D culture do not express LGR5.

Organoids grown from single isolated SSEA1$^+$ epithelial cells, developed in to gland-like spheroid structures after 10-14 days in 3D culture (as previously published) (Valentijn, Palial et al. 2013). The mature epithelial cells, forming these well-organised glandular structures, lost SSEA-1 expression we presumed to be associated with cellular differentiation, as described in the previous publication (Valentijn, Palial et al. 2013), and similarly, we could not identify any *LGR5* expression in any of the seven 3D cultures forming detectable organoid-structures studies (Figure 3.8).

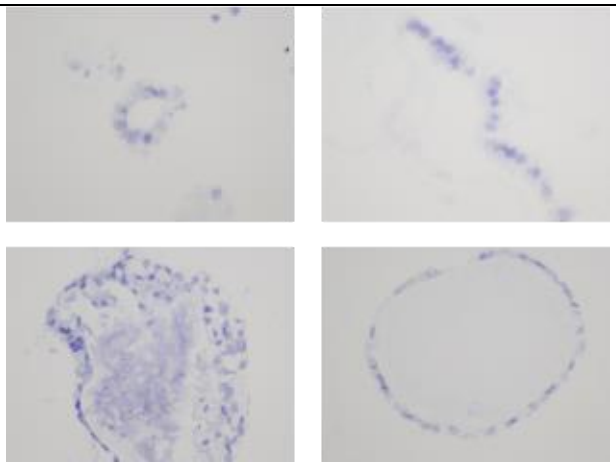


Figure 3.8 – **Epithelial organoids grown in 3D culture from SSEA1$^+$ cells did not express LGR5.** Representative *LGR5* ISH images of paraffin embedded 3D cultures of sorted SSEA1$^+$ epithelial cells grown for 14 days in Matrigel, clearly demonstrating gland-like organoid formation (All images x400).

3.4 **Discussion**

This is the first comprehensive study employing the gold standard method (ISH) in order to examine the cellular location of LGR5 expression in full thickness normal human endometrium. High *LGR5* expressing cells were seen in the endometrial LE, and in the basalis. Healthy human endometrium shows a dynamic, spatiotemporal, pattern of *LGR5* expression, suggesting

hormonal regulation. Endogenous and exogenous progestogens appear to inhibit *LGR5* expression in the endometrium, both *in vitro* and *in vivo.*

Previous evidence from other epithelial tissues proposes *LGR5* expression to be limited to stem cells and, thus, for *LGR5* to be an epithelial stem cell marker (Kumar, Burgess et al. 2014). We have shown that *LGR5* was not localised to a small number of cells in the adult endometrial basalis epithelium, which was the proposed location of the stem cell niche (Valentijn, Palial et al. 2013, Gargett, Schwab et al. 2016). The *LGR5* expression we observed in the human adult endometrium, unlike in the small intestine, mimics the *Lgr5* expression pattern seen in mouse uteri (Sun, Jackson et al. 2009). A uniform expression of *Lgr5* was seen in the ovariectomised uterine epithelium, and it is suggested that most of these remaining epithelial cells have the potential to proliferate, when necessary, for uterine glandular growth (Sun, Jackson et al. 2009). A mouse endometrial epithelial organoid system, which allowed long-term expansion of epithelium, also showed *Lgr5* gene expression (Boretto, Cox et al. 2017). In contrast, in humans, the whole of the endometrial functional layer is regularly shed with menstruation, a phenomenon not relevant to most mammals, including rodents. The initial step of embryo attachment and implantation occurs at the LE, which exists at a relatively distant location, in cellular terms, from the basalis (up to 16 mm in the mid-secretory phase). Due to external assaults, such as mechanical friction or infection, cells are continually lost and replaced from the surface of any epithelial tissue, including the skin, and intestine (Barker 2014); therefore, a similar daily cellular loss is likely to happen at the endometrial LE, which is exposed to the uterine cavity, and external environment. The daily maintenance of the LE may require locally positioned cells with progenitor ability. Supporting this hypothesis, rapid *Lgr5*$^{+}$ epithelial cell proliferation can be observed in many other organs upon tissue damage (Ng, Tan et al. 2014, Beumer and Clevers 2016).

We, therefore, hypothesise that it is possible for the human endometrium to have more than one epithelial stem/progenitor cell pool; one residing in the basalis (SSEA-1^{++} SOX9^{++} *LGR5*$^{+}$), supporting the massive regeneration of the functionalis after menstrual shedding or parturition; while the other

(*LGR5*$^{+\,+}$ SSEA-1^{+} SOX9^{+}) supports the embryo-implantation process, and maintains the LE cells that are likely to be lost on a daily basis (Figure 3.9). This is in agreement with the scanning electron microscopy (SEM) studies of human endometrium, the endometrial injury model of the rabbit (Ferenczy and Richart 1974), and neo-natal endometrial glandular development in humans (Cooke, Spencer et al. 2013). The persistent expression of the progenitor cell markers SOX9 and SSEA-1 in the LE, with concomitant high *LGR5* expression, corroborate further with the above hypothesis (Barker and Clevers 2010, Valentijn, Palial et al. 2013). Future work examining the functional properties of endometrial epithelial cell subpopulations that are isolated from the two anatomical regions within the human endometrium, which express either high or low LGR5, SOX9 and SSEA-1, is warranted.

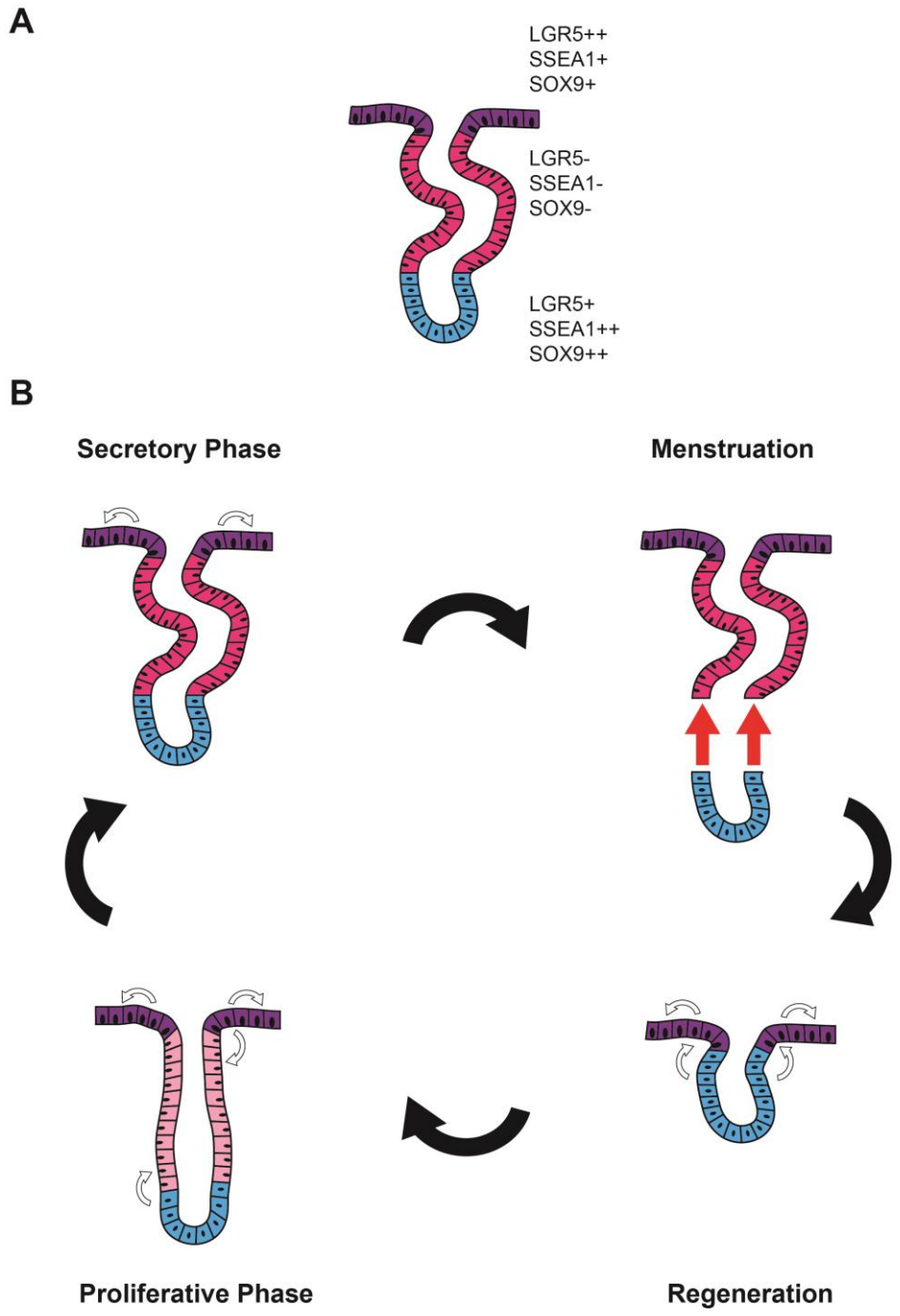


Figure 3.9 – **Putative model of endometrial epithelial regeneration**. A) The secretory phase endometrial epithelial configuration; LE contains *LGR5*$^{++}$ SOX9^{+} SSEA-1^{+} cells, functional glands contain *LGR5*$^{-}$SOX9^{-}SSEA-1^{-} epithelial cells and basalis glandular cells with SOX9^{++} SSEA-1^{++} *LGR5*$^{+}$ phenotype. B) Luminal and functional layers are shed at menstruation, LE

regenerated from the basalis epithelium (SOX9$^{+\,+}$ SSEA-1^{++} *LGR5*$^{+}$) after menstrual shedding, subsequently, the LE (*LGR5*$^{+\,+}$ SOX9^{+} SSEA-1^{+}) throughout the cycle regenerates itself and possibly also contributes to the regeneration of functional glands in the proliferative phase (*LGR5*$^{+}$ SOX9^{+} SSEA-1^{+}) whilst basalis glands are responsible for the regeneration of all/most of the epithelia of the functionalis in the proliferative phase.

N-cadherin has recently been identified as a possible endometrial epithelial stem cell marker, highlighting a sub set of epithelial cells at the basalis/myometrial junction (Nguyen, Xiao et al. 2017). Sorted N-cadherin^{+} epithelial cells were more clonogenic, underwent more population doublings (showing greater capacity for serial cloning), and differentiated into cytokeratin^{+} gland like organoids, when compared to the N-cadherin^{-} population (Nguyen, Xiao et al. 2017). During this work, I have not co-localised LGR5 with N-cadherin, however, with no evidence of N-cadherin in the LE, a high correlation between the two markers is unlikely.

The two antibody based studies examining LGR5 in the human endometrium (Gil-Sanchis, Cervello et al. 2013, Cervello, Gil-Sanchis et al. 2017) are in contrast to our work. We did not detect *LGR5* expression in the stroma, but only in the pancytokeratin expressing epithelial cells, and the observed proportion of epithelial cells expressing *LGR5* exceeded 1%. It should be noted that mRNA and protein levels may not necessarily correlate, and in our hands, the endometrial LGR5 protein expression, using two commercially available antibodies, demonstrated non-specific staining (Figure 3.10). Therefore, in agreement with the general consensus, we concluded that the reliability of antibodies against LGR5 remains in *considerable* doubt.

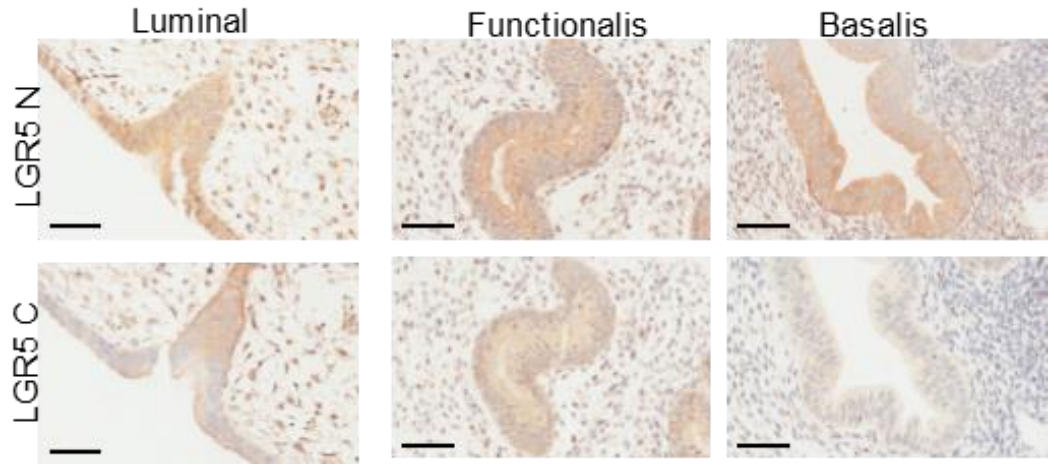


Figure 3.10 - Representative micrographs of the staining pattern demonstrated with 2 commercially available IHC *LGR5* antibodies, in the luminal, functionalis and basalis of the human endometrium (all images x400, scale bar =10 µm*)*.

Suppression of glandular regeneration and progenitor activity is postulated to occur within the progesterone-dominant, non-proliferative, secretory functional epithelium, where the lowest *LGR5* expression levels were observed.

The SSEA1^{+} epithelial cells grown in 3D culture have the architecture of functionalis epithelial glands, not primitive basalis-like glands. We postulate that this is the reason that they lose SSEA1 staining, and *LGR5* is also not expressed. The 3D organoid structures are formed from SSEA1 sorted epithelial cells, suggesting that these cells, possibly, do not contain many LE cells (SSEA1 low) to commence with, but mainly containing SSEA1^{++} (high) basalis cells.

In the absence of validated lineage markers for the various epithelial populations that are likely to exist within the endometrium, we cannot formally characterise the resident *LGR5*$^{+}$ cells as multipotent. Lineage tracing studies need to be completed in the human endometrial epithelium to identify the location of stem cells, this will further complement the *in vitro* functional studies, to confirm if *LGR5* expressing epithelial cells, indeed, represent the epithelial stem cell population (see Chapter 5).

Chapter 4. 3D Architecture of endometrial glands

4.1 Introduction

The current dogma is that endometrial epithelial stem cells residing in the terminal end of the basalis glands, at the endometrial/myometrial interface, are responsible for epithelial regeneration (Kim, Tavare et al. 2005, Valentijn, Palial et al. 2013, Gargett, Schwab et al. 2016). However, previous scanning electron microscopy (SEM) studies have suggested that endometrial re-epithelialisation occurs from remaining gland stumps and the luminal epithelium (LE) that is not shed in the cornual region, rather than the glandular bases (Ferenczy 1976). Although provision of transient amplifying (TA) cells to regenerate the functionalis glands at the gland stumps of the basalis/functionalis junctional region of the endometrium could be a direct function of the postulated stem cells residing in the gland bases, the theory cannot easily explain the cornual regeneration. Furthermore, this theory presumes endometrial glands to possess a single, blind-ended, linear, tubular architecture, where the terminal portion ends abutting the sub-endometrial myometrium junction (Figure 4.1). However, the precise three dimensional (3D) anatomical features, and micro-architectural organisation, of the human endometrial glandular epithelium is fundamentally unknown. In the context of conventional two dimensional (2D) histo-architecture, many have alluded to functionally different epithelial sub-compartments, located in luminal, functionalis, and basalis regions (Hild-Petito, Fazleabas et al. 1996, Nguyen, Sprung et al. 2012, Valentijn, Palial et al. 2013, Khan, Fujishita et al. 2016); yet distinct validated lineage markers for these various epithelial sub-populations do not exist. Traditional *in vivo* lineage tracing studies, in rodent models, that confirm the existence of an adult epithelial stem cell, are less relevant to the human endometrium because of its unique regeneration pattern (dissimilar to most other mammals) (Chan and Gargett 2006,

Cervello, Martinez-Conejero et al. 2007, Cervello, Gil-Sanchis et al. 2010, Cervello, Mas et al. 2011, Wang, Sacchetti et al. 2012).

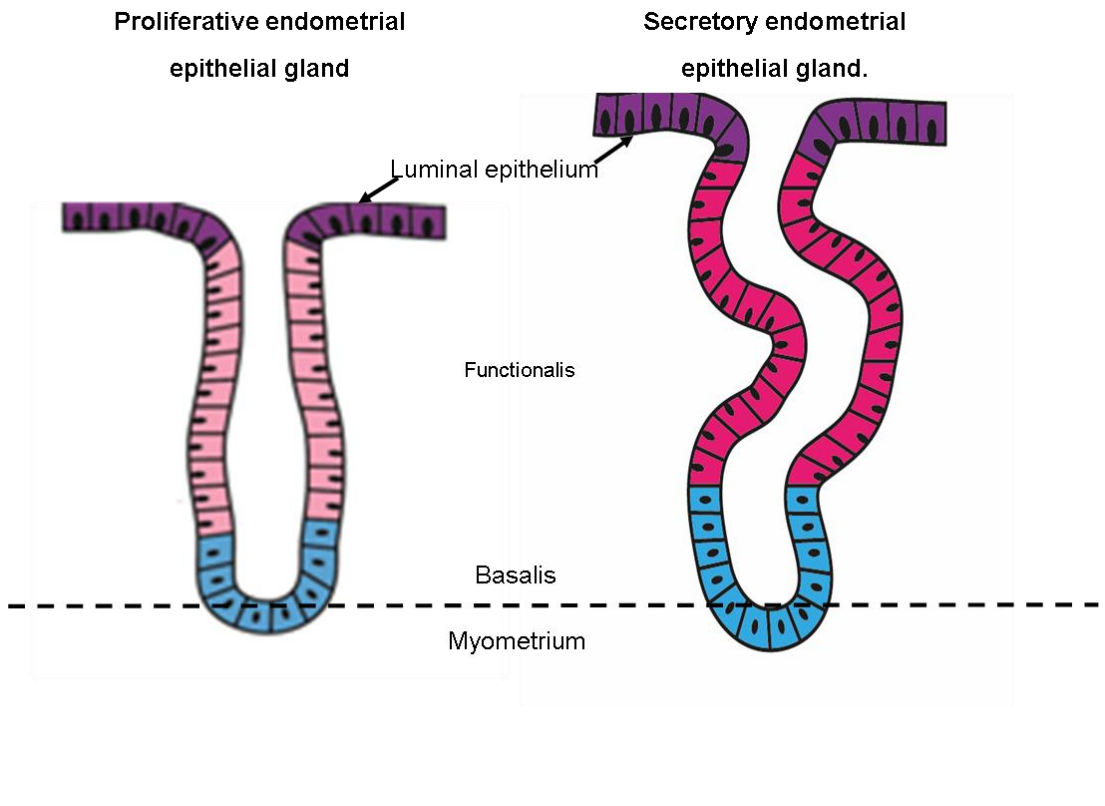


Figure 4.1 – Schematic demonstrating the current presumed endometrial glandular epithelial architecture, at different times of the menstrual cycle, a single blunt ended tube abutting the myometrium.

Attempts have been made to reconstruct endometrial epithelial cell lineages utilising two methods: Firstly, by analysing the non-random X chromosome inactivation with an X-linked androgen receptor gene, with DNA extracted from individual endometrial glands, the clonal constitution of glandular cells and luminal epithelium (LE) were examined to determine clonality of epithelial cells (Tanaka, Kyo et al. 2003); Secondly, by using methylation patterns to represent random replication errors, with the hypothesis that all daughter cells originate from a common ancestor, and cell divisions and ancestry may be surreptitiously recorded by identifying replication errors that naturally accumulate in a clock-like manner during aging (Tanaka, Kyo et al. 2003, Kim, Tavare et al. 2005). However, the methodology for these studies assumed a single, blunt-ended, tubular, 3D architecture for the glandular

epithelium, which is yet to be confirmed. Moreover, the sensitivity of the X-chromosome inactivation technique depends on the size, and tessellation, of X-inactivated patches, which in many tissues confounds the analysis of clonality (Novelli, Cossu et al. 2003).

3D tissue reconstruction, and examination of tissue at microscopic resolution from the digital images of serial sections, has significant potential to improve the understanding of the growth patterns, and the spatial arrangement, of diseased cells, and enhance the study of biomechanical behaviour of the tissue structures, towards better treatments (e.g. tissue-engineering applications) (Song, Treanor et al. 2013). Inparticular, diseases involving structural changes, or those in which the spatial relationship of disease features are important (Roberts, Magee et al. 2012). Its application to, or combination with, techniques, such as immunohistochemistry (IHC) or *in situ* hybridisation (ISH), adds further value by allowing understanding of additional phenotypic, or functional information (Roberts, Magee et al. 2012). The information is obtained by successively applying 2D image-to-image registrations, and then concatenating the set of aligned images, to form a 3D volumetric dataset (Song, Treanor et al. 2013).

Early 3D studies were performed by Wellings et al. in the late 1960s and 70s (Marcum and Wellings 1969, Wellings and Jensen 1973, Wellings, Jensen et al. 1975). Their methodology involved sectioning 50 to 150 consecutive 2 mm wide slices, staining them with hematoxylin and eosin (H&E), and sealing the sections in heavy polyethene bags in order to read the slices in the bags under the dissecting microscope, and mark the lesions on the overlying plastic. The lesions were cut out of the slices and subgross photographs were taken, before confirming findings with conventional histology. This method made selection and quantification of solitary lesions in whole mounts, possible for the first time, allowing the number of conventional paraffin embedded blocks needed to be examined to be reduced.

It is now possible to generate image stacks of serially sectioned tissues (Sun, Wang et al. 2009, Onozato, Klepeis et al. 2012), and in studies of pathological tissues. Such reconstructions have demonstrated previously

unknown architectural patterns, for example, showing that tubulo-lobular carcinomas are very similar to tubular carcinomas of the breast (Marchio, Sapino et al. 2006).

3D reconstruction has been undertaken of ductal carcinoma in situ (DCIS) of the breast, where it was anticipated that this would provide additional insight in to the natural course of the disease, and pathogenesis, on top of the normal 2D evaluation routinely performed. For example, if the disease is truly continuous in affected areas, or whether there is coexistent invasive disease; and whether multiple origins of invasion are the normal, or whether invasion usually occurs from a single point (Booth, Treanor et al. 2015). Initial applications of this technique have been mainly concerned with the investigations of the anatomical features, and microarchitecture of normal tissue (Kaufman, Brune et al. 1997), tumour invasion, growth factor expression, localisation of therapeutic targets in relation to microvasculature, and studying gene expression (for example, in developing mouse embryos (Han, van Hemert et al. 2011), and the developing human brain) (Wang, Lindsay et al. 2010).

There are competing alternative techniques to 3D tissue reconstruction using individually stained serial sections, and these include: Alternative non-destructive 3D imaging techniques, consisting of; optical projection tomography (Quintana and Sharpe 2011); 3D imaging with ultrasonography (Prager, Ijaz et al. 2010); microscopic magnetic resonance imaging or X-ray microcomputed tomography; confocal laser scanning or multiphoton microscopy, and serial block face imaging (e.g., episcopic fluorescence image capture and high-resolution episcopic microscopy) (Denk and Horstmann 2004). These methods are still in the process of being optomised, so could potentially have benefits over 3D tissue reconstruction with serial sections. Conventional histopathological staining and interpretation techniques have significant, and obvious, advantages over other techniques (Roberts, Magee et al. 2012).The Leeds Institute of Cancer and Pathology has previously employed 3D histopathological techniques to reconstruct a variety of tissues (including liver and kidney), providing detailed visualisation of structural features, and spatial relationships (Roberts, Magee et al. 2012).

We aimed to test the current hypothesis that the endometrial epithelial glands are blind ending, singular tubes, running from the LE to the endometrial/ myometrial junction.

4.1.1 The Research Questions that were addressed

Main research question: What is the 3D histo-architectural arrangement of the human premenopausal endometrial epithelial compartment?

We aimed to answer the above question in 2 steps:

1. What is the 3D structural organisation of the premenopausal endometrial functionalis glands?
2. What is the 3D structural organisation of the premenopausal basalis glands?

4.2 Methods

4.2.1 Patient population

Patients who donated endometrial tissue are detailed in Table 4.1. Three, full thickness, endometrial samples were collected from patients undergoing hysterectomy for benign conditions at Liverpool Women's Hospital, from 2009-2017. The hysterectomy specimens were opened immediately after surgical excision, and full thickness wedge biopsies, containing all endometrial layers and subendometrial myometrium were taken. These tissue sections were fixed for 24 hours in 4% (v/v) buffered formalin, then paraffin embedded for histochemical staining. The patient demographic details were documented at the time of sample collection. None of the patients received hormonal treatments for 3 months prior to surgery, and they did not have known endometrial pathology.

Sample Number	Age	BMI	Parity	Smoker	Stage of Cycle
1	42	22.3	2	N	Proliferative
2	44	18.7	0	N	Secretory
3	44	29.4	2	N	Proliferative

Table 4.1 - Demographical details of the patients involved in the study.

4.2.2 Histochemistry

From each block, one hundred sequential 4 µm thick serial sections were cut and numbered with the same orientation throughout. The slides were H&E stained as follows: dewaxed (as per chapter 2), followed by staining firstly in Gills 2 Haematoxylin (Thermo Scientific, Runcorn, UK) for four minutes, rinsing in tap water, and dipping in acid alcohol, before returning to tap water for five minutes. Slides are then placed in 70% alcohol, followed by 95% alcohol for one minute each. Counterstain with Eosin Y (Thermo Fisher Scientific, Runcorn, UK) for four minutes, and rinse in water, before dehydrating, clearing, and mounting in synthetic resin.

4.2.3 Model generation

The 100 consecutive stained slides (H&E), of full thickness endometrium, were scanned using an Aperio ScanScope slide scanner (Aperio Technologies, Vista, CA, USA) at x400 magnification, creating virtual slides. The slides were sent to the Leeds Institute of Cancer and Pathology where they underwent the process of `registration` aligning in stacks, so that the 2D features were aligned, to form smooth 3D topographies. This registration resulted in a stack of images which had been aligned, thus, could be rendered in 3D as a 3D ‘volume’. This ‘volume’ was a 3D image, which was analogous to a cube of glass containing the H&E stained tissue. The virtual slides were registered using Slice Registration Application (SliceRegApp) program, [version 11.1.1 (64 bit OpenMP build), University of Leeds, Leeds, UK]. For this, a reference image was selected in the middle of the image

stack, and was used to align subsequent images, proceeding out from the centre, aligning all images to their neighbours (Video 4.1). This aligned each virtual slide to adjacent slides within the dataset, before we uploaded the images into the FreeD16 240 software program (Andrey and Maurin 2005) for 3D reconstruction, as follows: Serial images (TIFF file format) were imported into FreeD software v 1.10 image stack files; Endometrial gland boundaries in a specific area on the individual sections of full thickness endometrium were drawn manually in each 2D serial image, and connected along the third dimension between adjacent slides, producing 3D models.

Video 4.1 – Representative video of one of the 100 consecutively sectioned, H&E stained aligned, endometrial tissue blocks.

The different anatomical areas of the full thickness endometrial samples (the basalis and the functionalis) were examined in greater detail, relating to the architecture of the glands, and the gland interactions.

4.3 **Results**

3D reconstructions of full thickness endometrial samples did not show a single, tubular, blunt-ended architecture for the human endometrial glands.

We were able to create 3D reconstructions from all 3 full thickness endometrial samples using the 100 consecutive 2D sections (Figure 4.2). This, therefore, provided the basis for progression on to further work in order to analyse the samples, examining their regional specific 3D configuration.

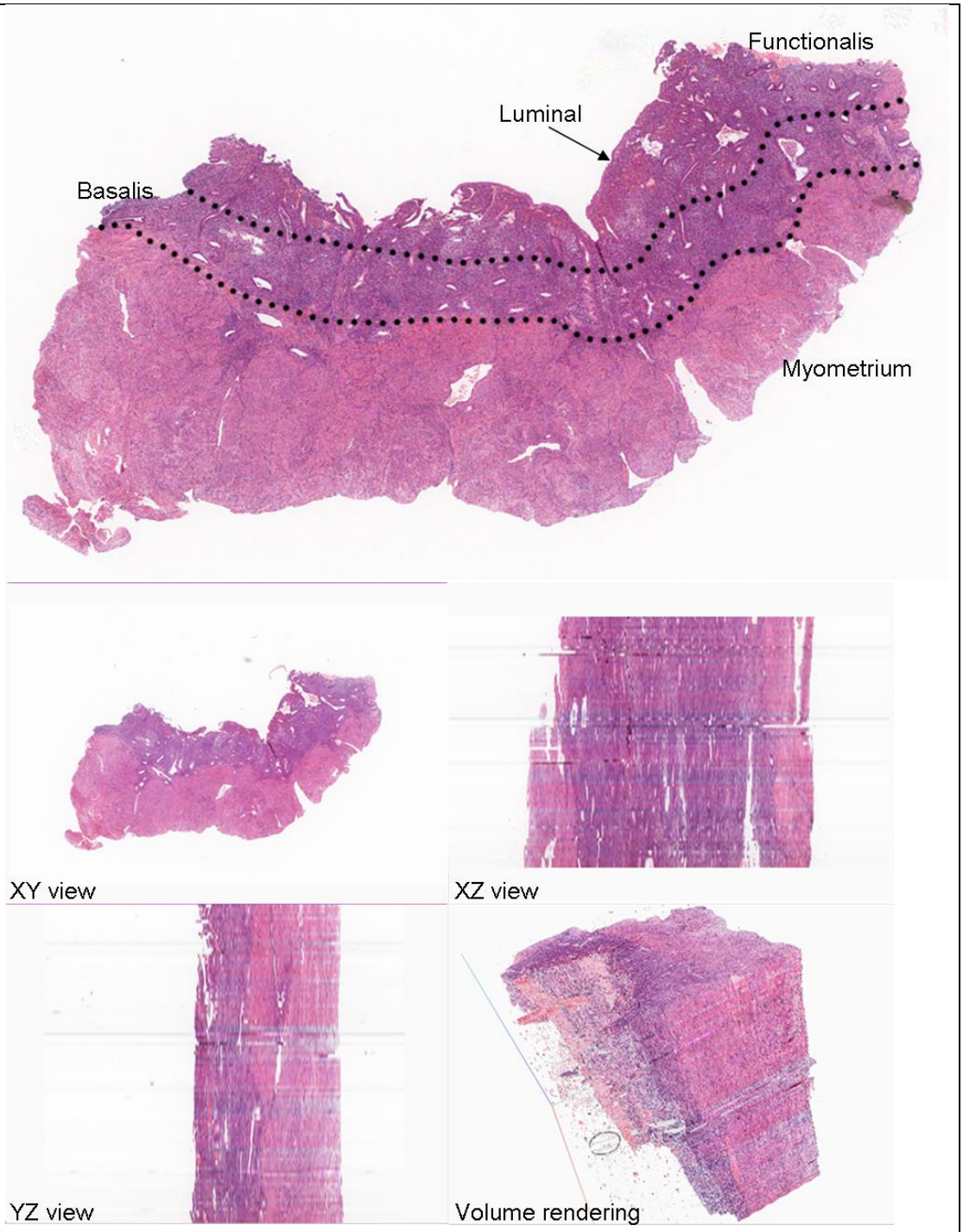


Figure 4.2 – Representative images of one of the full thickness endometrial samples formed into a 3D block.

Premenopausal functionalis glands are non-branching and run a parallel, vertical course through the superficial human endometrium.

The endometrial functionalis glands could be tracked from the functionalis/basalis junction to the LE, running parallel to each other (Figure 4.3 A and B, 4.4 A and B and 4.5 A and B, and videos 4.2, 4.3 and 4.4). The proliferative glands were thinner, straighter, and easier to trace (Figure 4.3 A and B and 4.4 A and B) than the much longer, coiled secretory glands, with wider lumens (Figure 4.5 A and B).

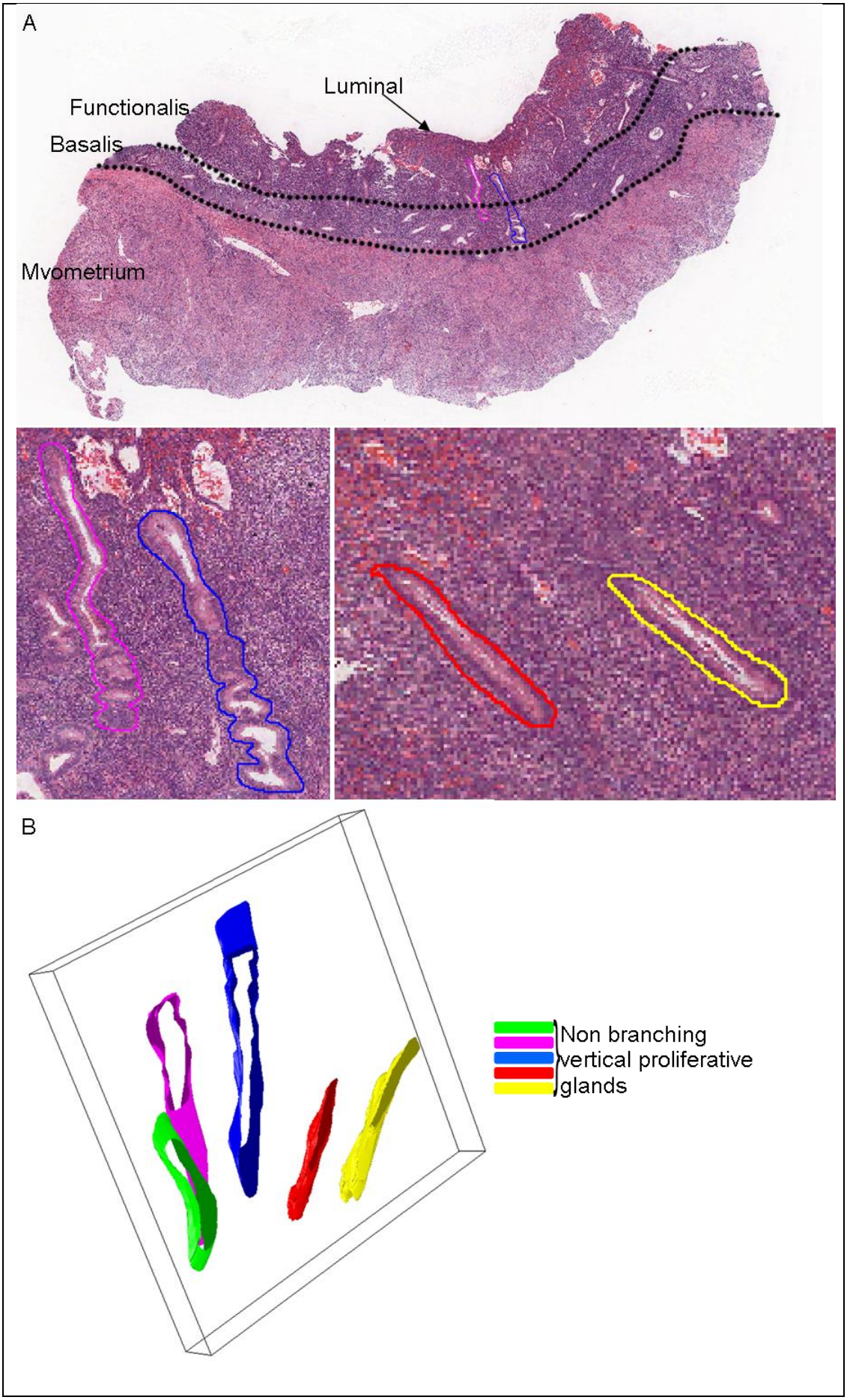
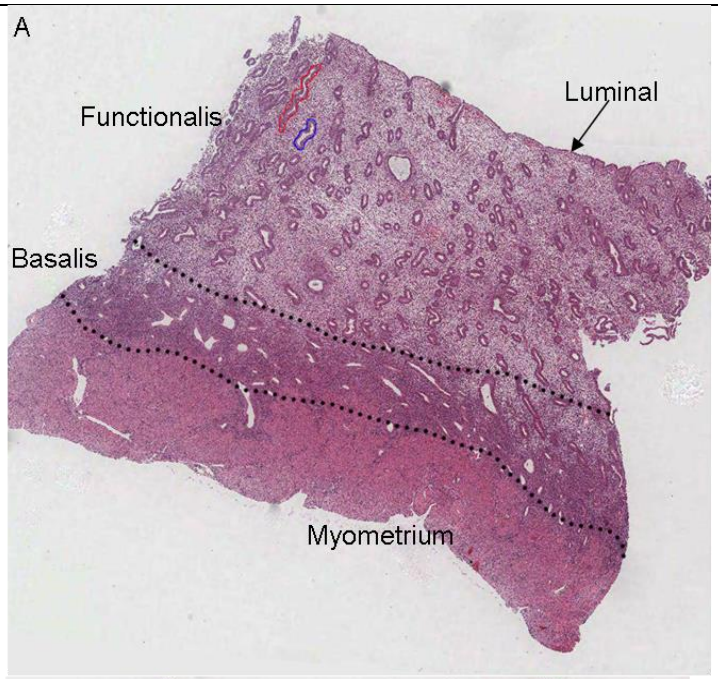
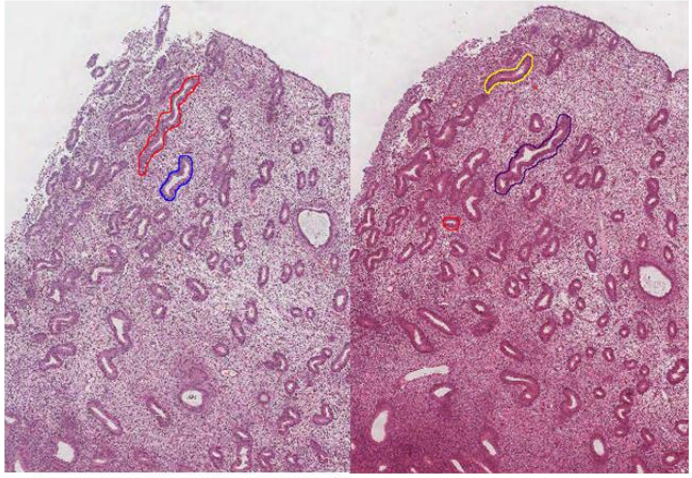

A
Luminal
Functionalis
Basalis
Myometrium
B
Non branching
vertical proliferative
glands

Figure 4.3 – **Demonstration of premenopausal early-proliferative functionalis glands are non-branching and run a parallel, vertical course through the superficial human endometrium.** (A) Representative micrographs of full thickness proliferative endometrium showing functionalis glands running parallel to each other. (B) 3D schematic of vertical non branching functionalis glands.


A
Functionalis
Luminal
Basalis
Myometrium



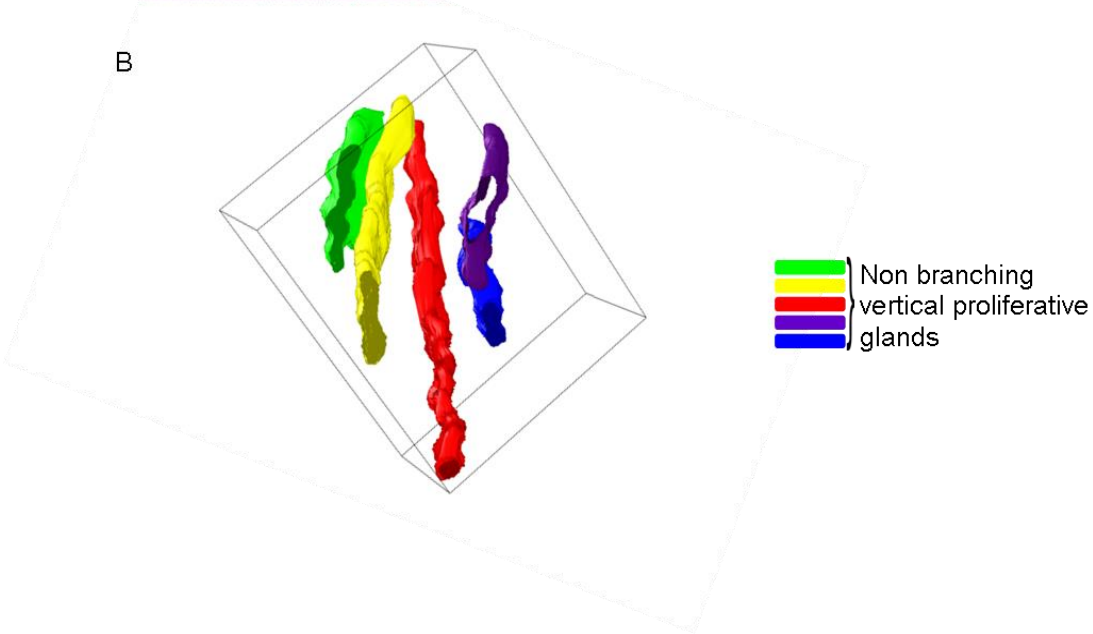

B
Non branching
vertical proliferative
glands

Figure 4.4 - **Demonstration of premenopausal mid-proliferative functionalis glandular 3D-architecture.** (A) Representative micrographs of full thickness proliferative endometrium showing functionalis glands run a parallel, vertical course through the superficial functionalis human endometrium (B) 3D schematic of vertical non branching functionalis glands.

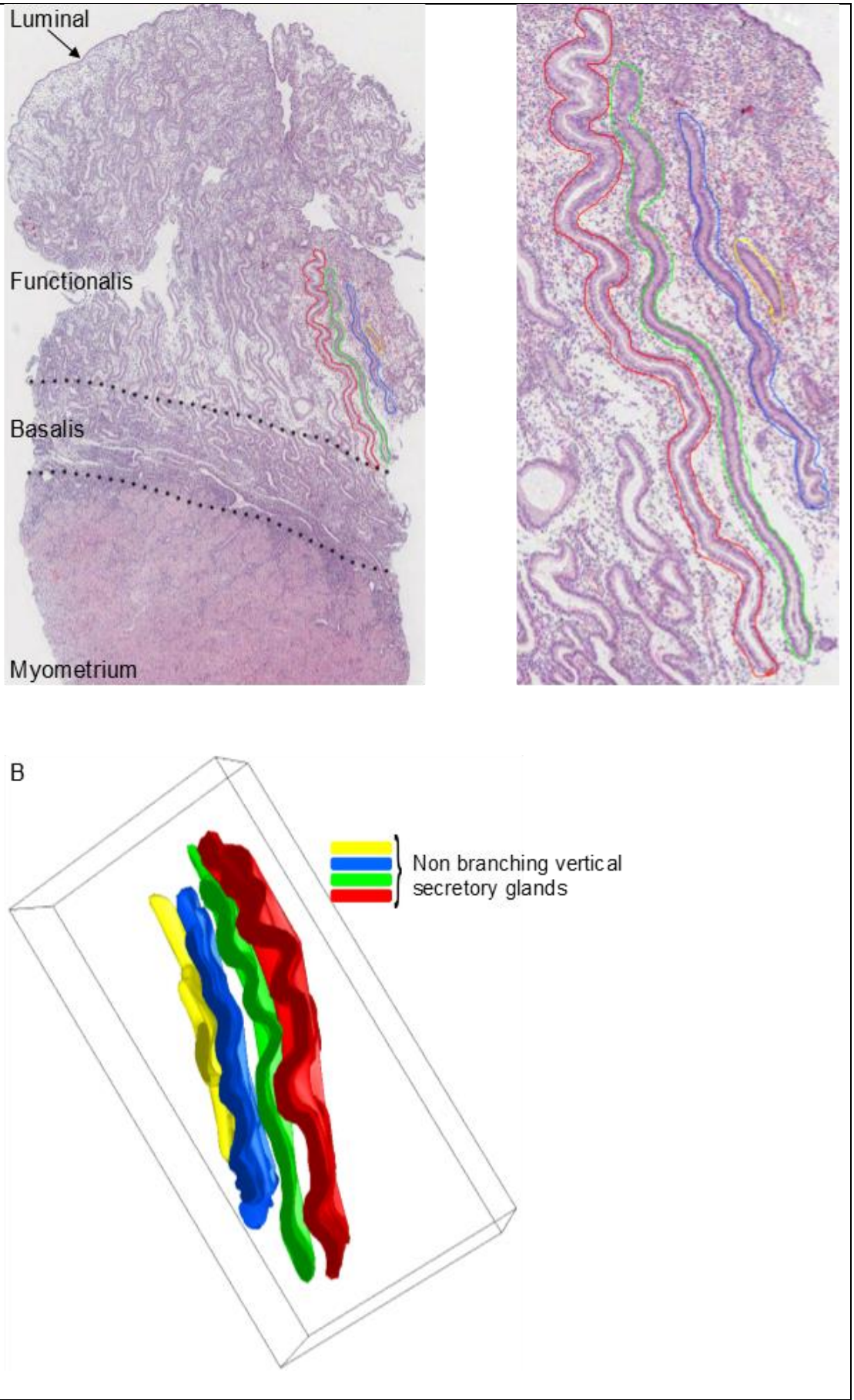

Luminal
Functionalis
Basalis
Myometrium
B
Non branching vertical
secretory glands

Figure 4.5 – **Demonstration of premenopausal secretory functionalis glands are also non-branching and run a parallel, vertical course through the superficial human endometrium.** (A) Representative micrographs of full thickness proliferative endometrium showing functionalis glands running parallel to each other. (B) 3D schematic of vertical non branching functionalis glands.
Video 4.2 – **Non-branching vertical early proliferative functionalis glands**. 3D video documenting the non-branching vertical early-proliferative phase functionalis glands.
Video 4.3 - **Non-branching vertical mid-proliferative functionalis glands.** 3D video documenting the non-branching vertical mid-proliferative phase functionalis glands.
Video 4.4 - **Non-branching vertical secretory functionalis glands.** 3D video documenting the non-branching vertical secretory phase functionalis glands

Premenopausal endometrial basalis glands form a complex and branching configuration, proceeding in a horizontal course to the myometrium.

The endometrial basalis glands were similarly traced from the sub-endometrial myometrium, up to the endometrial functionalis/basalis junction. These basalis glands demonstrated a more complex, and a dissimilar, structural organisation when compared with the parallel, singular vertical functionalis glands. They often branched, as well as enveloping around one another horizontally in a root –like “stolon” configuration, giving rise to single lumen functionalis glands arranged at 90 degree angles to them (Figure 4.6 A and B, 4.7 A and B and 4.8 A and B, and videos 4.5, 4.6 and 4.7). Since the stem cells are postulated to reside in them, this basalis glandular organisation is likely to provide an effective glandular propagation strategy to maintain continuous endometrial covering of the uterine cavity, and its scar-less regeneration.

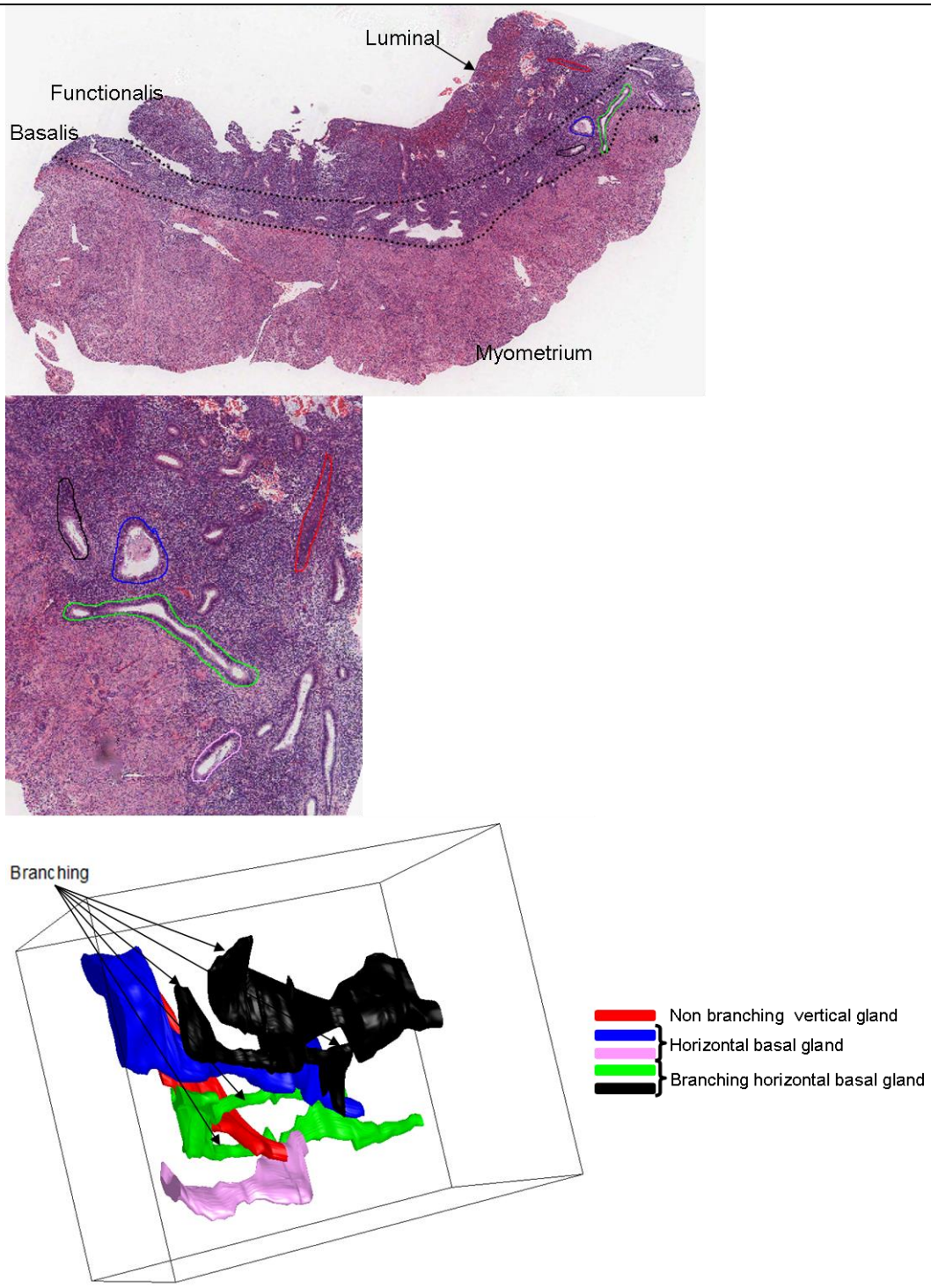


Figure 4.6 – **Demonstration of premenopausal endometrial basalis glands forming a complex and branching configuration, proceeding in a horizontal course to the myometrium.** (A) Representative micrographs of full thickness early-proliferative endometrium showing branching basalis glands running at a horizontal course to the myometrium. (B) 3D schematic of branching horizontal basalis glands.

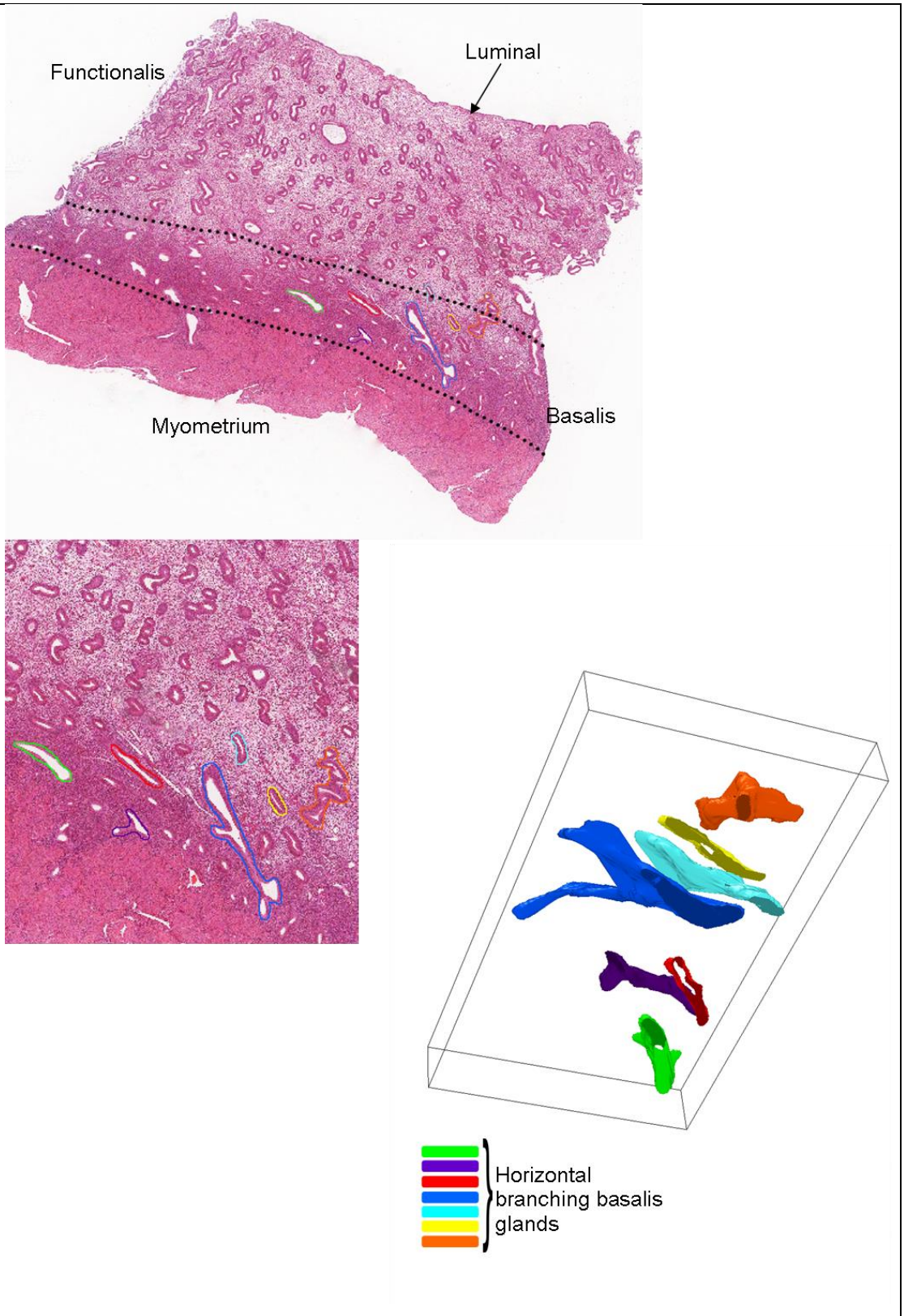


Figure 4.7 – **Demonstration of premenopausal endometrial basalis glands forming a complex and branching configuration, proceeding in a horizontal course to the myometrium.** (A) Representative micrographs

of full thickness mid-proliferative endometrium showing branching basalis glands running at a horizontal course to the myometrium and enveloping around one another. (B) 3D schematic of branching basalis glands and a non branching functionalis gland rising vertically from the basalis.

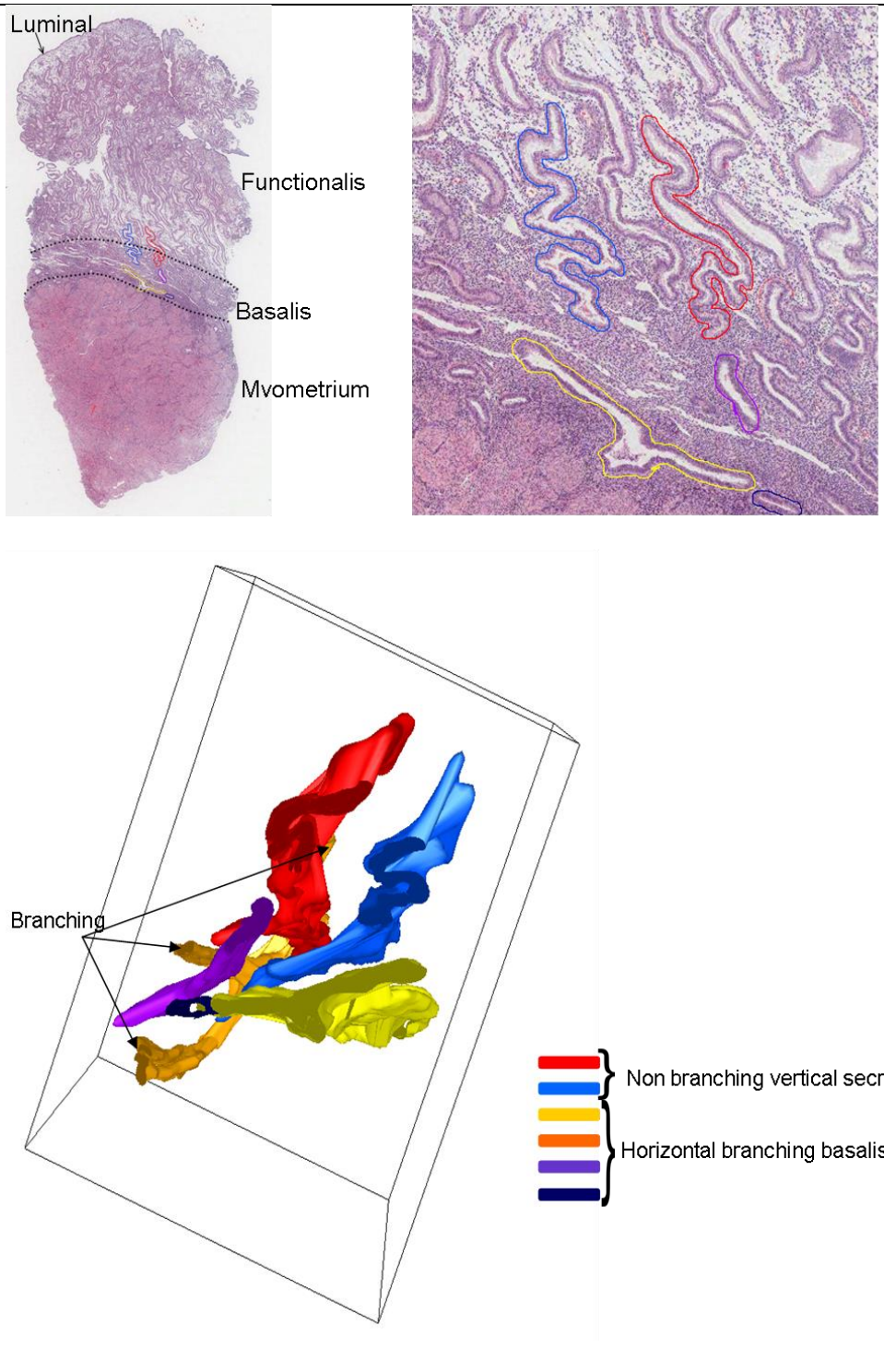


Figure 4.8 – **Demonstration of premenopausal endometrial basalis glands forming a complex and branching configuration, proceeding in a horizontal course to the myometrium.** (A) Representative micrographs of full thickness secretory endometrium showing branching basalis glands running at a horizontal course to the myometrium and enveloping around one another. (B) 3D schematic of branching basalis glands and non branching functionalis glands rising vertically from the basalis.
Video 4.5 – **Horizontal branching basalis glands.** 3D video documenting the branching early-proliferative basalis glands.
Video 4.6 - **Horizontal branching basalis glands.** 3D video documenting the branching mid-proliferative basalis glands.
Video 4.7 - **Horizontal branching basalis glands and vertical non branching functionalis glands.** 3D video documenting the branching secretory basalis glands and non branching functionalis glands.

4.4 **Discussion**

The 3D architecture of the human adult endometrial glands was previously uncharacterised until now, and 3D reconstruction work has not been previously attempted on full thickness endometrial samples containing the whole glandular element. The current consensus had been that the endometrial glandular arrangement is similar to intestinal glandular crypts, assuming a single, blunt ended, ductular design. The novel work presented in this chapter gives a whole new insight into the complex, previously unknown, intricate, 3D histoarchitectural organisation of the human endometrial glands, replacing the existing, obsolete, schematics of the endometrial glandular organisation (Figure 4.9 A and B).

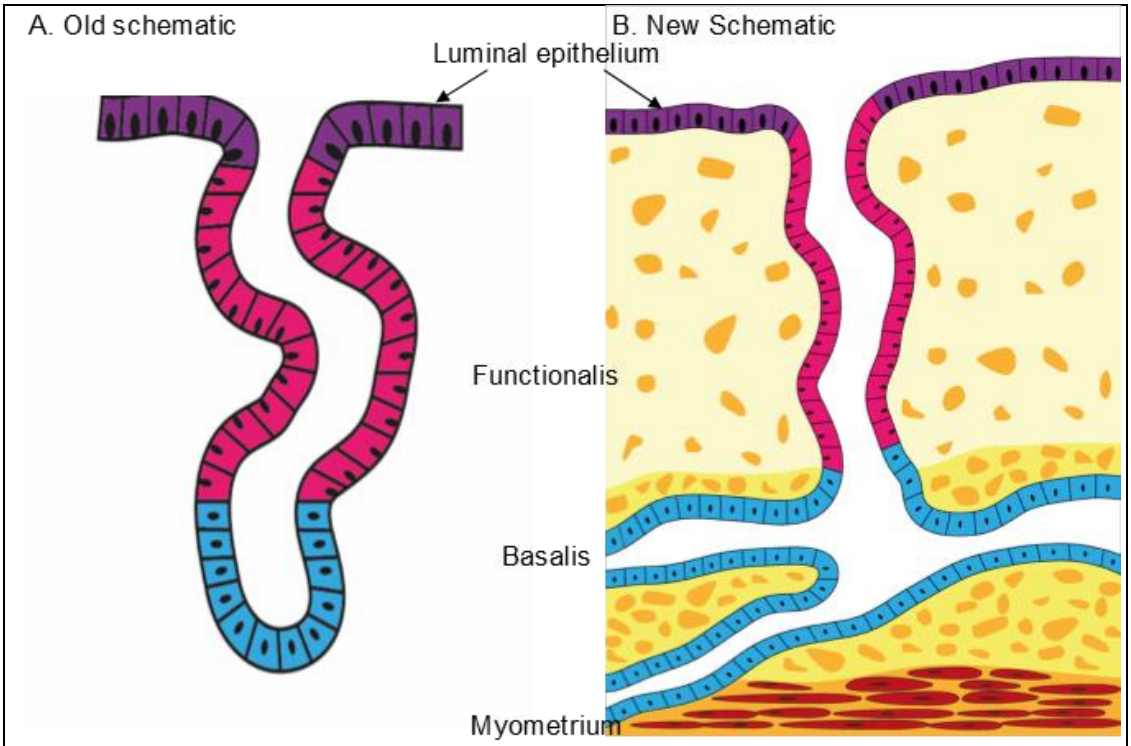


Figure 4.9 – **Endometrial premenopausal secretory functionalis glands are non-branching and run a parallel, vertical course through the superficial human endometrium whereas the basalis glands form a complex and branching configuration, proceeding in a horizontal course to the myometrium.** (A) Old schematic of endometrial glands. (B) Proposed new schematic of endometrial glands with complex basalis architecture.

The functionalis glands, which are shed on a monthly basis, were found to run parallel to each other, without branching in their course, from the LE to the functionalis/basalis junction. Although these glands assumed a single tubular configuration, like the straight colonic crypts (Henrikson R 1997), their spiralling and coiling rendered them to be more structurally complex than the straight intestinal crypts. Furthermore, the spiralling increased with advancing phase, and associated cellular differentiation, of the menstrual cycle (from proliferative to secretory) in the functionalis glands.

Based on observations made on 2D tissue sections, others have reported the basalis glands of the human endometrium to run a horizontal course

alongside the myometrium (Ferenczy and Richart 1973, Cooke, Spencer et al. 2013), but their suggestions have been overlooked in all recent manuscripts, and frequent schematics have adopted the intestinal crypt-like basalis gland configuration, with blind ending tubes resting into the myometrium (Gargett, Schwab et al. 2016). This work, the first 3D reconstructions of the human pre-menopausal endometrial glandular compartment, agrees with previous studies (Ferenczy and Richart 1973, Cooke, Spencer et al. 2013) and offers an alternative schematic (Figure 4.9). The glands have a more complex/intricate course than a blind ending tube, likely to be assisting in the self-preserving, self-renewing, scar-less nature of the human endometrial regeneration. The regular endometrial regeneration on a monthly basis is well known (Hapangama, Kamal et al. 2015), and it also regenerates following parturition, iatrogenic destruction after surgical ablation, and curettage. Therefore, relying on a single blind ending tube may not be the most efficacious arrangement, as the stem cell rich, basalis, glandular ends could potentially be easily lost/artificially removed during one of the above situations, leading to the loss of the regenerative ability of the endometrium in the near vicinity. Therefore, the preservation of the potential endometrial epithelial stem cell niche, the basalis glands, is vital. The complex, root-like stolen configuration, therefore, provides the ideal rapid replenishment of the stem cell niche, in any area of the basalis glandular element.

This work forms the basis for a new avenue of study, to understand many unanswered questions relevant to human embryo-implantation, utilising the 3D architecture (similar to the recent mouse studies) which demonstrated previously unknown information in murine embryo-implantation. Yuan et al recently showed, using 3D reconstruction, a previously unknown event in implantation biology; that LE invaginates to form a crypt with the existing glands, setting up a direct communication between the blastocyst and glands within the crypt (Yuan, Deng et al. 2018). Aberrations of these events were captured in a 3D context in the *Vangl2*-deleted epithelia, providing compounding evidence for the role of epithelial Vangl2/PCP signalling in

implantation (Yuan, Deng et al. 2018). Similarly, the work presented in this chapter paves the way for further 3D studies.

Further modelling of different human endometrial samples needs to be completed, scrutinising closely the LE, postmenopausal (PM) endometrium, and endometrial pathologies; such as pre-cancerous endometrial hyperplasia, endometrial cancer, and endometriosis. The LE, as previously discussed, should be seen as a separate entity to the functionalis and basalis epithelium. With the current 3D modelling data presented in this chapter, the dynamic architecture of the LE is difficult to discern. Further work to tease out the relationship between the LE and the functionalis glands in the superficial functionalis layer needs to be completed; potentially, digitally re-slicing the re-constructed tissue blocks, or forming models utilising different markers of different epithelial areas to distinguish interactions in more detail, would be future avenues to explore. PM samples are obviously required to be interrogated, to confirm, or dispute, the current consensus that these glands are similar to the basalis glands of the premenopausal endometrium in their nature, and architecture. This method could also be utilised to allow us a better understanding of the interplay between normal endometrium, hyperplasia, and cancer; and to distinguish if women with particular endometrial pathologies (such as recurrent miscarriage, endometriosis, subfertility, or heavy menstrual bleeding) carry an altered architectural arrangement to women without endometriosis.

Chapter 5. *In vivo* lineage tracing using mitochondrial DNA mutations

5.1 Introduction

All adult organs have stem cells that are responsible for their maintenance and repair. The characteristics, behaviour, and 'undifferentiated' status of a stem cell changes when it is removed from its niche. The identification of these long-lived adult stem cells (ASCs) in a solid organ is, therefore, particularly challenging, and only certain techniques are suitable for isolating/identifying stem cells of human organs. This chapter describes *in vivo* lineage tracing, the "gold standard" method of stem cell identification.

Lineage analysis is a technique originally developed to study early embryos, and represents, by far, the most powerful and reliable tool for identifying and confirming stem cells; and for deciphering other aspects of tissue behaviour (Fox DT 2009). The methods employed for lineage tracing in animals are very different to the methods that can be used for lineage tracing in humans, due to the obvious unacceptability/unsuitability for studies using genetic modification, and ingestion of fluorescent probes to be undertaken in human subjects. However, *in vivo* lineage tracing where the progeny of a stem cell is directly tracked/identified in intact tissue is the ultimate confirmation of the existence of a stem cell. The method used in the experiments described in this chapter enables lineage tracing *in vivo* in the intact human endometrial tissue, without potential harm to the participating subjects. This method involves tracing mitochondrial DNA (mtDNA) mutations in the epithelial compartment, and is deemed a natural experiment since genetic changes occur spontaneously, allowing lineages to be tracked and, in several instances, the stem cell niche to be identified (Wright 2012).

Taylor and colleagues, in Newcastle-upon-Tyne (Taylor, Barron et al. 2003), initially used this method of tracing mtDNA mutations in human colon tissue. Subsequently, it was generalised by Fellous et al for the identification of

human epithelial stem cell niches in solid organs, to study liver (Fellous, Islam et al. 2009), intestine, pancreas, and skin (Fellous, McDonald et al. 2009). We have employed this method, for the first time, to prove the existence of human endometrial epithelial stem cells. The added value of this method is that the analysis of passenger mutations has proven to be more useful than the analysis of driver mutations, because maintenance of passenger mutations is presumably not subject to selective pressure (Fearon and Bommer 2011).

Mitochondria are essential intracellular organelles that are required for oxidative phosphorylation, and are found in all nucleated human cells. They contain the only non-chromosomal DNA in human cells, their own mtDNA (Taylor and Turnbull 2005). The mitochondria are, therefore, under the dual control of nuclear and mtDNA (Taylor, Barron et al. 2003). High levels of reactive oxygen species (ROS) production (Richter, Park et al. 1988), lack of protective histones, and the absence of DNA repair mechanisms to protect mtDNA, renders the mitochondrial genome more prone to mutations than nuclear DNA (Taylor, Taylor et al. 2001, He, Chinnery et al. 2002, Fellous, McDonald et al. 2009). Therefore, mtDNA displays elevated levels of spontaneous, non-pathogenic, passenger mutations, and it is through these that the visualisation and following of the progeny of an individual stem cell in a tissue section has been realised, as these passenger mutations act as clonal markers (Walther and Alison 2016). The passenger mutations are passed on to daughter cells during cell division, providing a platform for studying cell lineage in human tissues.

Mitochondrially-encoded cytochrome C oxidase (CCO) is an enzyme forming the last step of the electron transport chain in respiratory complex IV (Mootha, Lepage et al. 2003, Taylor, Barron et al. 2003, Greaves, Preston et al. 2006, Li, Park et al. 2006, McDonald, Greaves et al. 2008). It is a major regulatory site for oxidative phosphorylation and is essential for the assembly and respiratory function of the enzyme complex (Li, Park et al. 2006). Spontaneous passenger, non-pathogenic, mutations can occur in the CCO gene, and they confer little or no selective growth advantage to normal cells (Nooteboom, Johnson et al. 2010). Importantly, such mutations are readily

detectable in tissue sections, using dual-colour enzyme histochemistry (Fellous, McDonald et al. 2009), or immunohistochemistry (IHC) (Greaves, Preston et al. 2006).

When CCO mutations occur, they can affect all copies of the mitochondrial genome within a cell, homoplasmy, or there may be a mixture of mutated and wild-type genomes in the same cell, heteroplasmy (Lightowlers, Chinnery et al. 1997, Taylor and Turnbull 2005). In the presence of heteroplasmy, identification of a detectable deficiency in the CCO enzyme requires at least 80% of the CCO genes in a cell to be mutated (Sciacco, Bonilla et al. 1994, Taylor, Barron et al. 2003).

The expansion of a mutation within a cell can follow a stochastic course, which, over time, can evolve from a heteroplasmic state to a homoplasmic, or near homoplasmic, state. This process is termed `genetic drift` (Elson, Samuels et al. 2001, Wright 2012). The mutation spreads, firstly, within the individual mitochondrial genome, and then in the mitochondria of the stem cell, to a level of homoplasmy. Therefore, due to this lengthy process, wholly-mutant crypts are rarely seen before the age of 40 (Greaves, Preston et al. 2006), indicating the time required for near-homoplasmy to be reached (Greaves, Preston et al. 2006, Wright 2012). For the same reason, it is assumed that only stem cells live long enough to acquire the near-homoplasmic state, enabling their detection of a biochemical deficiency (Fellous, McDonald et al. 2009).

In mucosal tissues, such as the small intestinal crypt and the gastric gland, CCO^- crypts and glands were shown to contain cells of all lineages, demonstrating that all of the cells in these glands originated from a CCO^- common ancestor, a stem cell (Wright 2012). This method has, subsequently, been utilised to confirm the existence of epithelial stem cells in the colon (Taylor, Barron et al. 2003), pancreas (Fellous, McDonald et al. 2009), bladder (Gaisa, Graham et al. 2011), and prostate (Gaisa, Graham et al. 2011), but has not yet been utilised in the human endometrium. The work described in this chapter is, therefore, intended to extend the method to,

conclusively, examine the existence of a human endometrial epithelial stem cell.

5.1.1 The research questions that were addressed

1. Do CCO mutations occur in the human endometrium?
2. What is the natural history of endometrial CCO mutations in the human endometrium with advancing age?
3. Do all epithelial cells from a CCO negative patch contain the same mtDNA mutation, confirming clonality, and proving the existence of endometrial epithelial stem cells?
4. Does more than one stem cell contribute to functionalis endometrial gland regeneration?
5. Do CCO-deficient clonal patches incorporate cells of all epithelial regions (basal, functional and luminal), therefore demonstrating multipotency?
6. Can 3D modelling of CCO-deficient clonal patches identify the location of the endometrial epithelial stem cell niche?

5.2 Methods

5.2.1 Patient population

Patient groups who donated endometrial tissue are detailed in Table 5.1. 78 full thickness endometrial samples were collected from patients undergoing hysterectomy for benign conditions at Liverpool Women's Hospital, from 2009-2017. The hysterectomy specimens were either opened immediately after surgical excision (70/78), or transported straightaway on ice to the pathology laboratory at the Royal Liverpool University Hospital, to be opened, and sectioned, by the pathologist (8/78) (allowing a larger sample); and full thickness wedge biopsies containing all endometrial layers, and subendometrial myometrium were taken. These tissue sections were processed in two ways: fixed for 24 hours in 4% (v/v) buffered formalin, then paraffin embedded for IHC staining; flash frozen with liquid nitrogen, and stored at -80°C for enzyme histochemistry, laser capture micro dissection

(LCM), polymerase chain reaction (PCR), and Sanger sequencing of the CCO gene. The patient demographic details were documented at the time of sample collection. None of the patients received hormonal treatments for 3 months prior to surgery, and they did not have known endometrial pathology.

Age	BMI	Parity	Smoking
21	25.9	0	N
23	23.5	0	N
25	20.2	0	Y
27	23	3	N
31	24.9	3	N
32	27.8	2	Y
32	26.6	2	N
34	23.8	3	N
35	30.5	0	N
35			N
37	41.5	3	N
37	26.5	2	Y
37	21.7	4	Y
38	30.8	3	N
38	27.9	4	N
39	32	2	Y
39	27.9	1	N
39	26.5	2	N
40	34.7	3	Y
41	30.7	1	N
41	26.7	4	Y

41	23.2	1	Y
42	23.5	2	N
42	22.3	2	N
43	40.5	3	N
43	28.6	2	N
43	24.5	0	N
43	26.7	2	N
44	29.4	2	N
44	29.6	1	N
44	24.3	2	Y
44	32.3	1	N
44	38	2	N
44	30.7	0	N
44	24.5	2	N
44	18.7	0	N
45	21.7	4	Y
46	23.1	3	N
46	26.8	5	N
47	29.8	2	N
47	30.2	3	N
47	21.6	2	N
47	32.4	5	N
47	25	1	N
47	23.4	1	N
47	25.8	2	N

47	29.4	2	N
48	29.9	4	N
48	40.1	3	N
49	24.4	2	N
49	23.2	0	N
49		2	Y
49	28.4	2	N
49	27.6	3	N
50	20.3	3	N
52	24.5	2	N
52	26.3	2	N
57	30.8	2	N
58	20	2	N
60	31	2	N
60	26.9	5	Y
61	27.7	4	N
62	28.3	3	N
62	32.2	4	N
62	27.4	1	N
65	28.8	3	Y
65	18.5	2	N
66	24.9	3	N
69	24.7	4	N
69	26.8	3	N
72	35.8	3	N

74	35.6	3	N
74	32	3	N
75	29.6	2	N
75	29.6		N
76	25.2	2	N
77	26.6	2	N
78	26.8	4	N

Table 5.1 – Demographical details of the patients involved in the study.

5.2.2 Immunohistochemistry

After antigen retrieval in citrate or TRIS buffer (see antibody conditions, Table 5.2) (as previously described in chapter 2), 4 µm formalin fixed paraffin embedded (FFPE) sections were blocked with 3% hydrogen peroxide for 10 minutes, then immunostained with the appropriate antibody for the given time, and dilution in Table 5.2. This was followed by incubation for 30 minutes at RT in the appropriate secondary antibody. Detection was with ImmPRESS polymer based system, and visualisation was with ImmPACT DAB (Vector Labarotories, Peterborough, UK) (used as per manufacturer's instructions). Sections were counter stained in Gill 2 Haemotoylin (Thermo Scientific, Runcorn, UK), dehydrated, cleared, and mounted in Consul-Mount. Matching isotype replaced the primary antibody as a negative control, with internal positive control in each staining run.

Primary Antibody	Type	Clone	Supplier	Antigen Retrieval	Dilution	Incubation	Secondary
SSEA-1	Monoclonal	MC-480	Biolegend	Tris 3 mins	1:800	Overnight at 4ºC	Mouse
Pancytokeratin	Monoclonal	C2562	Sigma	Citrate 2 mins	1:4000	Overnight at 4ºC	Mouse
N Cadherin	Monoclonal	8C11	Abcam	Citrate 2 mins	1:200	Overnight at 4ºC	Mouse
SOX 9	Polyclonal	SOX 9	R&D	Citrate 4 mins	1:400	Overnight at 4ºC	Mouse
CK5/6	Monoclonal	D5/16B4	Merck	Tris 3 mins	1:250	Overnight at 4ºC	Mouse
MUC 1	Monoclonal	BC-2	Serotec	Citrate 2 mins	1:400	Overnight at 4ºC	Mouse
BCAM	Monoclonal	EPR4164	Abcam	Citrate 2 mins	1:100	Overnight at 4ºC	Rabbit
CCO	Monoclonal	1D6E1A8	Invitrogen	Citrate 2 mins	1:1000	30mins room temperature	Mouse
Ki67	Monoclonal	Ki67	Dako	Citrate 4 mins	1:200	Overnight at 4ºC	Mouse

Table 5.2 – IHC Antibody details.

Immunostaining was analysed with specific reference to CCO presence in the luminal epithelium (LE), the functionalis (glands in the upper two-thirds of the endometrium below the LE), and the basalis (glands in the lower one-third of the endometrium adjacent to the endo-myometrial junction), in numbers of wild type vs partial gland vs complete gland mutations.

5.2.3 *In situ* hybridisation

ISH for *LGR5* expression was performed as, previously described (Baker, Graham et al. 2015), on 4 µm sections using the RNAscope 2.5 High Definition Brown assay, according to the manufacturer's instructions

(Advanced Cell Diagnostics, Hayward, CA). Samples were baked at 60°C for 1 hour 30 minutes, followed by de-paraffinisation and incubation with Pretreat 1 buffer, for 10 minutes at RT. Slides were boiled in Pretreat 2 buffer for 15 minutes, followed by incubation with Pretreat 3 buffer, for 15 minutes at 40°C. Slides were incubated with the relevant probes for 2 hours at 40°C, followed by successive incubations with Amp 1 to 6 reagents. Staining was visualized with DAB for 20 minutes, then lightly counterstained with Gill's haemotoxylin. RNAscope probes used were *LGR5* (NM_003667.2, region 560-1589 catalogue number 311021), *POLR2A* (positive control probe) (NM_000937.4, region 2514-3433 catalogue number 310451), and dapB (negative control probe) (EF191515, region 414-862, catalogue number 310043).

LGR5 expression was quantified according to the five-grade scoring system recommended by the manufacturer, previously described (Baker, Graham et al. 2015) (0 = No staining or less than 1 dot to every 10 cells (40× magnification), 1 = 1–3 dots/cell (visible at 20–40× magnification), 2 = 4–10 dots/cell, very few dot clusters (visible at 20–40× magnification), 3 = > 10 dots/cell, less than 10% positive cells have dot clusters (visible at 20× magnification), 4 = > 10 dots/cell, more than 10% positive cells have dot clusters (visible at 20× magnification)).

5.2.4 Enzyme histochemistry

12 µm sections of frozen full thickness endometrium were cut with the cryostat and incubated, firstly in CCO medium (100 mM cytochrome c, 4 mM diaminobenzidine tetrahydrochloride, and 20 µg/mL catalase in 0.2 M phosphate buffer, pH 7.0 all from Sigma Aldrich, Poole, UK) at 37°C for 50 minutes to allow for detection of CCO activity in brown, followed by washes in PBS pH7.4 (3 × 5 minutes). Succinate dehydrogenase (SDH) medium incubation followed (130 mM sodium succinate, 200 µM phenazine methosulphate, 1 mM sodium azide, 1.5 mM nitroblue tetrazolium in 0.2 M phosphate buffer, pH 7.0) at 37°C for 40 minutes before further PBS washes (3x 5 minutes). Following this, the sections were allowed to air dry for LCM, or dehydrated in a graded ethanol series (70%, 95%, 2 × 100%), cleared in

Histoclear (Fisher Scientific, Leicestershire, UK), and mounted in DPX (BDH Laboratory Supplies, Poole, United Kingdom). This method, therefore, allowed CCO-positive cells (wild type) to be stained brown, and CCO-deficient areas to be highlighted, and recognised, using a second stain detecting the activity of a nuclear encoded enzyme, succinate dehydrogenase (SDH, blue, a component of complex II of the respiratory chain).

5.2.5 Isolation of total DNA from micro-dissected endometrium

Fresh frozen sections (12 µm) of endometrium were mounted on PALM membrane slides (Carl Zeiss, Oberkochen, Germany), underwent dual enzymatic histochemistry (as described above) and were air-dried for one hour. Single endometrial epithelial cells of interest (mutated and wild type) and stroma (for control) were cut into sterile 0.5 mL AdhesiveCap PCR tubes (Carl Zeiss, Oberkochen, Germany) (previously ultraviolet (UV) treated) using a PALM Microbeam laser capture system (Carl Zeiss, Oberkochen, Germany). After LCM, 15 µL of ATL buffer was added to each sample to commence cell lysis (RNA extraction of the samples was done immediately after LCM, or after storing at -20°C overnight).

5.2.6 DNA extraction

DNA was extracted using Qiagen QIAamp DNA Micro kits. 10 µL of proteinase K and 15 µL of ATL buffer was added to the tubes already containing the tissue, and this was mixed by pulse vortexing for 15 seconds, before incubating for 3 hours at 56°C, with occasional agitation. 25 µL of buffer ATL was added, and 50 µL of buffer AL, before mixing by pulse vortexing for 15 seconds. To ensure efficient lysis it is essential that the sample and buffer AL are thoroughly mixed to yield a homogenous solution. 50 µL of 100% ethanol was added, and this was mixed thoroughly by pulse vortexing for 15 seconds, before incubating for 5 minutes at RT. The mixture was briefly centrifuged to remove drops from inside the lid, and the entire

lysate was transferred to the QIAamp MinElute column (in a 2 mL collection tube) without wetting the rim. The tube was centrifuged at 6000g for 1 minute, and the QIAamp MinElute column was placed in a clean collection tube (discarding the collection tube and flow through). 500 µL of buffer AW1 was added, and the mixture was centrifuged at 6000g for 1 minute. The QIAamp MinElute column was placed in a clean collection tube, and 500 µL of buffer AW2 was added. Centrifuge was performed at 6000g for 1 minute, again, and the MinElute column was placed in a clean 2 mL collection tube before centrifuging at full speed (20,000g) for 3 minutes to dry the membrane completely (this step is necessary as ethanol carryover may interfere with some downstream applications).

The QIAamp MinElute column was placed in a clean 1.5 mL microcentrifuge tube, and 20 µL of distilled water was applied. The mixture was incubated for 5 minutes before spinning on maximum for 1 minute, and adding a further 20 µL of water, incubating for a second 5 minutes, and spinning at maximum for 1 minute.

5.2.7 Mitochondrial DNA sequencing of individual endometrial epithelial cells

The entire sequence of the mitochondrial genome from micro-dissected endometrial cells was determined using the single cell lysate as the DNA template, and a two stage nested PCR amplification protocol. The primary PCR reactions involved amplification of the mitochondrial genome in 9 fragments, of approximately 2 kb, using a series of overlapping primer pairs. These initial large PCR reaction products decrease the risk of amplifying pseudogenes when extracting DNA from small quantities of DNA. All PCR amplifications were performed in a 25 µL volume containing 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl, 0.001% wt/vol gelatin), 0.2 mM dNTPs, 0.6 µM primers, 1 U AmpliTaq Gold DNA polymerase (Applied Biosystems), and 1 µL single-cell lysate. Reaction conditions were 95°C for 10 minutes, and 38 cycles of 94°C for 45 seconds; 58°C for 45 seconds and 72°C for 2 minutes. The final extension was for 8 minutes,

followed by incubation at 4°C indefinitely. The secondary PCR reactions involved amplification of the primary PCR products with 28 primer pairs, specifically to generate overlapping fragments of between 600–700 base pairs that span the entire sequence of the human mitochondrial genome. Reaction conditions were 95°C for 10 minutes, and 30 cycles of 94°C for 45 seconds; 58°C for 45 seconds and 72°C for 1 minute. The final extension was for 8 minutes, followed by incubation at 4°C indefinitely. To facilitate the direct sequencing of PCR-amplified products, all primer pairs are tagged with M13 sequence and designed to anneal optimally at 58°C. All reactions proceeded for 30 cycles and used 2 µL of primary reaction product as DNA template. PCR products were treated with ExoSaP-IT according to the manufacturer's protocol, before EDTA-ethanol cleaning, then Sanger sequencing reaction using BigDye 3.1 terminator cycle-sequencing chemistries (Applied Biosystems) on an ABI 3730XL automated DNA sequencer.

The sequences obtained were analysed using 4Peaks software (www.mekentosj.com), together with Clustal W2 software (EMBL-EBI), and compared to the revised Cambridge reference sequence (Andrews, Kubacka et al. 1999), with sequences from stromal controls and CCO normal specimens, to identify polymorphisms and somatic mutations from the CCO-deficient sequences. Individual mutations were confirmed in all instances by repeating the first and second-round PCRs, and resequencing the products.

5.2.8 CCO model generation

The 100 consecutive CCO stained slides of full thickness endometrium were scanned using an Aperio ScanScope slide scanner (Aperio Technologies, Vista, CA, USA) at ×400 magnification, creating virtual slides. The slides were sent to the Leeds Institute of Cancer and Pathology where they underwent the process of `registration` aligning in stacks, so that the 2D features in them are aligned to form smooth 3D topographies. This registration resulted in a stack of images which had been aligned, thus, could be rendered in 3D as a 3D 'volume'. This 'volume' was a 3D image, which was analogous to a cube of glass, containing the H&E stained tissue. The

virtual slides were registered using Slice Registration Application (SliceRegApp) program, [version 11.1.1 (64 bit OpenMP build), University of Leeds, Leeds, UK]. For this, a reference image was selected in the middle of the image stack, and was used to align subsequent images proceeding out from the centre, aligning all images to their neighbours. This aligned each virtual slide to adjacent slides within the dataset, before we uploaded the images into FreeD16 240 software program (Andrey and Maurin 2005) for 3D reconstruction, as follows: Serial images (TIFF file format) were imported into FreeD software v 1.10 image stack files; The mutations were manually annotated in every section; and these were super imposed onto one of the pictures to visualise the location of the mutant clones.

5.3 **Results**

CCO-deficient epithelial patches exist in the endometrium.

The screening of human tissues for CCO-deficient cells can initially be performed on FFPE tissue sections using a mouse monoclonal, anti-Oxphos complex IV subunit 1 antibody, and IHC, clearly identifying cases of interest which contain CCO-deficient areas.

The presence of cells with common ancestry in the human endometrium was determined by screening the endometrial tissue sections with IHC for CCO activity (brown), which confirmed the presence of blue CCO-deficient glands (Figure 5.1). 75 FFPE endometrial samples from women aged 21-78 years were screened using IHC, and CCO-deficient patches (defined as any CCO-negative gland, partial, or complete) were observed in 58 of the samples (77.3%).

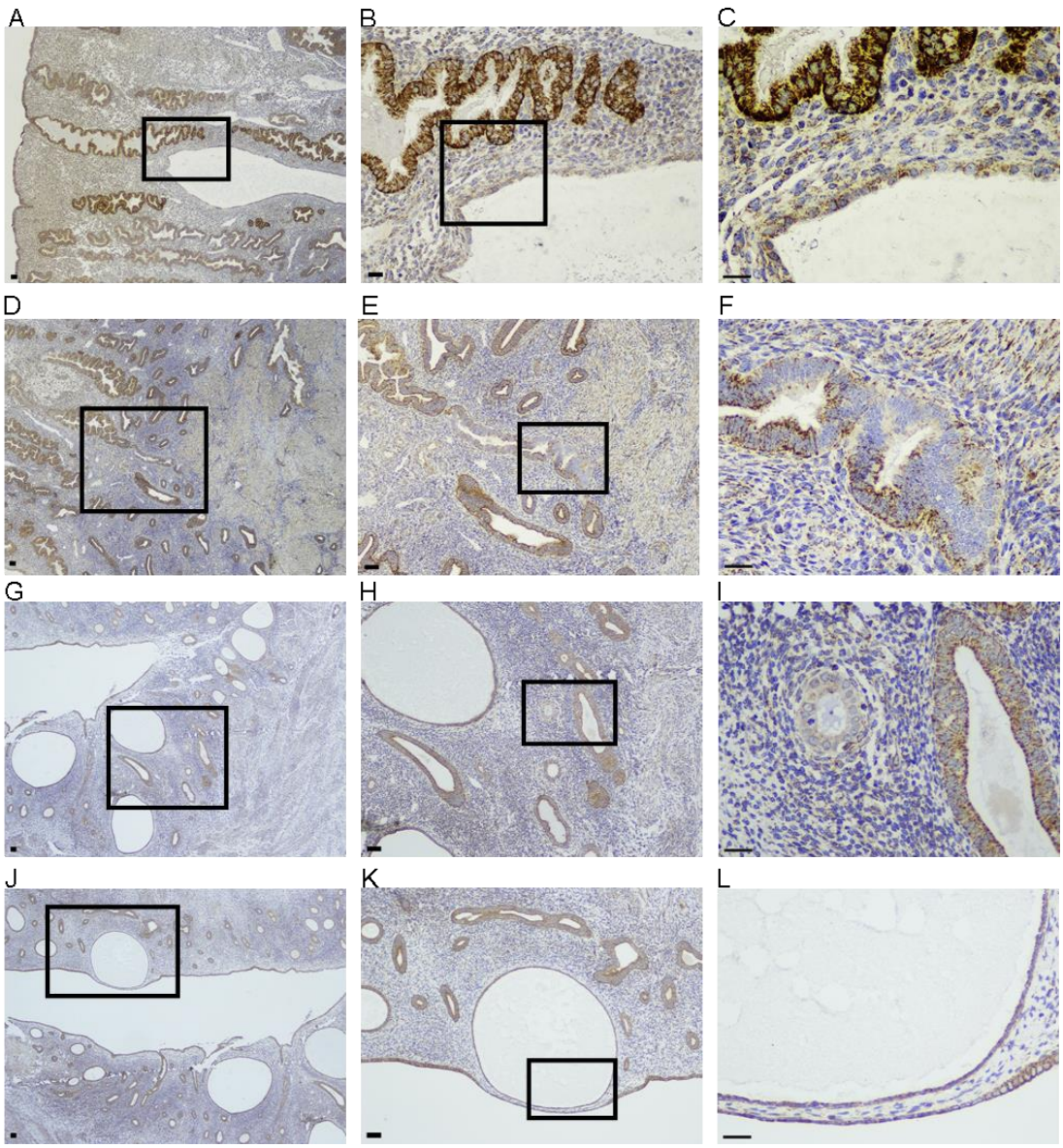


Figure 5.1 - **CCO-deficient epithelial patches exist in the human endometrium** (A,B and C) Representative micrographs of pre menopausal functionalis epithelium with CCO deficiency. (D,E and F) Representative micrographs of premenopausal basalis epithelium with CCO deficiency. (G,H and I) Representative micrographs of PM epithelium with CCO deficiency. (J,K and L) Representative micrographs of PM LE with CCO deficiency. (Images A,D,G and J x40 magnification , images B,E,H and K x100, C,F,I and L x400, all scale bars = 10 μm*).*

Incidence of CCO-deficient epithelial patches increases across the reproductive years, up to the age of menopause.

The presence of CCO-deficient clonal patches in the endometrial samples examined appeared to increase up to menopausal age, but plateaued after that (figure 5.2A). The lowest prevalence of clonal patches was observed in the endometrium from 20-29 year old women (50% (2/4)), and all endometrial samples examined from women aged 60-69 years contained CCO-deficient glands (100% (10/10)). However, beyond that age, the prevalence of these patches remained stable, despite advancing age in the endometrium of PM women.

The percentage of partially-deficient, and wholly-deficient, glands as a percentage of the total glands present in the sample, also increases with age (Figure 5.2B and 5.2C), as does the percentage of the partially-deficient glands in the basalis layer (Figure 5.2D).

When the pre-menopausal functionalis layer of the endometrium (only present in premenopausal women, and regenerated monthly) was considered, the presence of wholly-deficient functionalis glands (Figure 5.2E), and partially-deficient functionalis glands (Figure 5.2F) increased across the decades, with none being present in the 20-30 year olds (neither partial nor complete). The earliest appearance of a CCO-negative clonal patch was observed in the basalis glands of a 25 year old, suggesting the long lived epithelial cells (likely stem cells) to be located in the basalis.

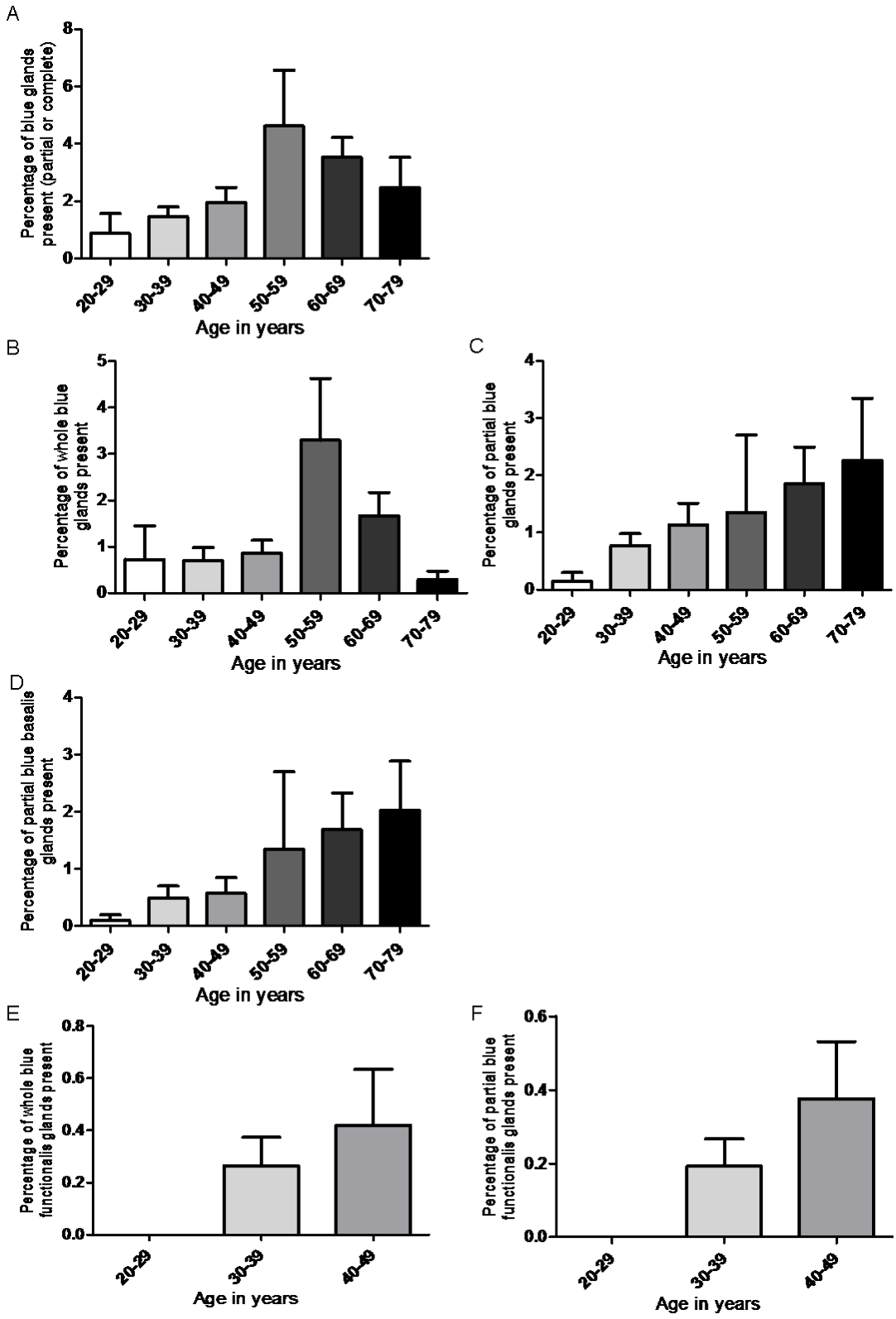


Figure 5.2 – ***The incidence of CCO-deficient epithelial patches increases across the reproductive years up to the age of menopause.*** (A) The presence of CCO-deficient clonal patches in the endometrium increases up until the age of menopause (n=78). (B) The percentage of wholly CCO-

deficient clonal patches increased with age until menopause (n=78). (C) The percentage of partially CCO-deficient glands increases with age (n=78). (D) The percentage of partially CCO-deficient glands in the endometrial basalis glands increases with age (n=78). (E) The percentage of wholly CCO-deficient glands in the pre-menopausal functionalis glands increases with age (n=52). (F) The percentage of partially CCO-deficient pre-menopausal functionalis glands increases with age (n=52).

When all samples were considered, age, or BMI, did not correlate significantly with the prevalence of clonal patches.

When all endometrial samples derived from the nulliparous women were considered against all multiparous women (any ages), nulliparous women had more (although not significantly) CCO-deficient patches.

Unique mitochondrial mutations confirm the existence of endometrial epithelial stem cells in the endometrium.

Eight frozen full thickness endometrial samples were screened with enzyme histochemistry for their suitability for isolating mtDNA from single cells using LCM and sequencing of the mtDNA. In order to prove the clonality of endometrial glands, we selected an endometrial sample containing both CCO-deficient glands (Figure 5.3 A&B), as well as partially mutated glands (Figure 5.3 C&D) (Taylor, Barron et al. 2003).

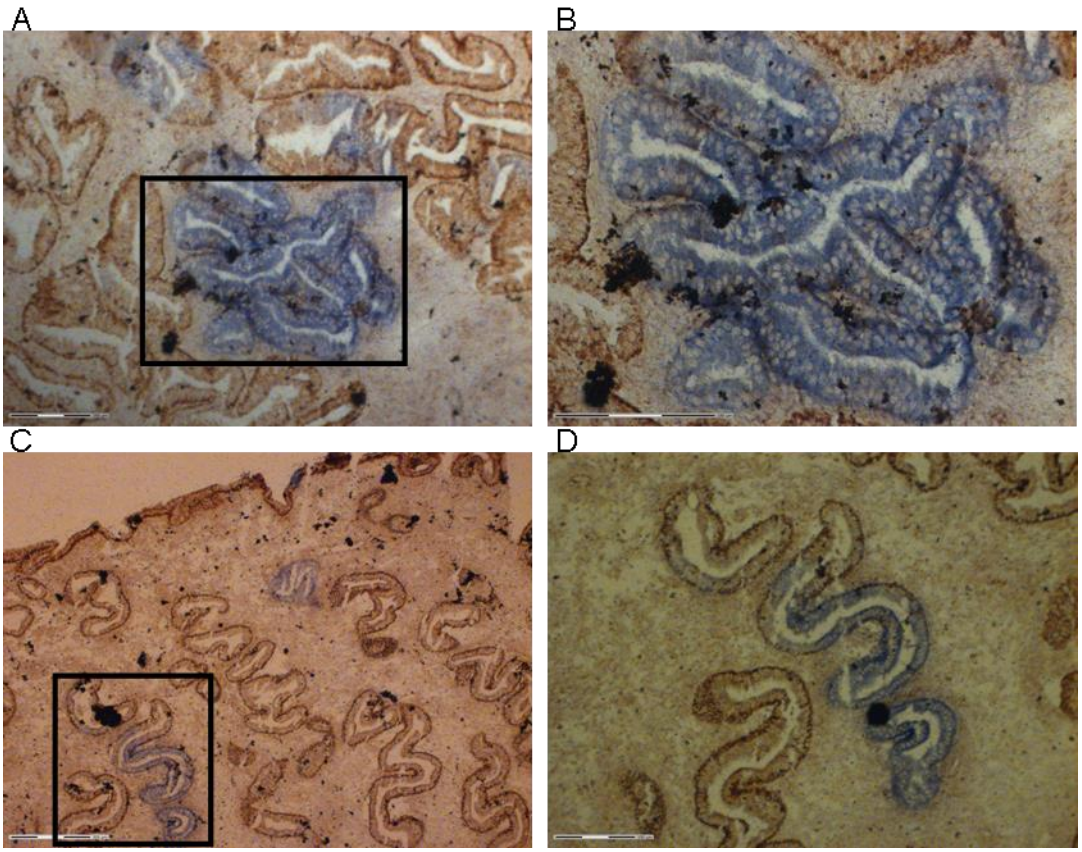


Figure 5.3 – **Dual enzyme histochemistry demonstrates CCO deficiency and partially mutated glands in the endometrium.** (A and B) Representative micrographs showing a wholly CCO-deficient endometrial basalis gland. (C and D) Representative micrographs showing a partially CCO-deficient endometrial functionalis gland. (Image A x200, scale bar = 150 µm, Image B x400, scale bar = 150 µm, Image C x40, scale bar = 300 µm, Image D x200, scale bar = 150 µm).

To demonstrate that patches of CCO-deficient cells represent *bona fide* clonal expansions (similar to the previous studies in other solid organs), multiple CCO-deficient (blue) cells were isolated using LCM alongside isolating multiple wild type (brown) cells and stroma (as control tissue) (Figure 5.4). Their entire mtDNA genome was sequenced to reveal common somatic mutations that would indicate a common cell-of-origin. Figure 5.5 shows that all of the micro dissected cells from the CCO-deficient area of a partially mutated gland contained the same mtDNA mutation (6667 C to T), which was not present in the surrounding CCO-proficient cells,

demonstrating a clonal expansion. Previous work has informed us that the chance of the same mutation occurring separately in two neighbouring cells (if they are not from the same progenitor cell) is calculated to be $2.48x10^{9}$ (Lin, Lim et al. 2010). Therefore, this data conclusively confirms the existence of an endometrial epithelial stem cell, whilst the existence of partially mutated glands suggest that at least some endometrial glands are regenerated by more than one epithelial stem cell (polyclonal origin).

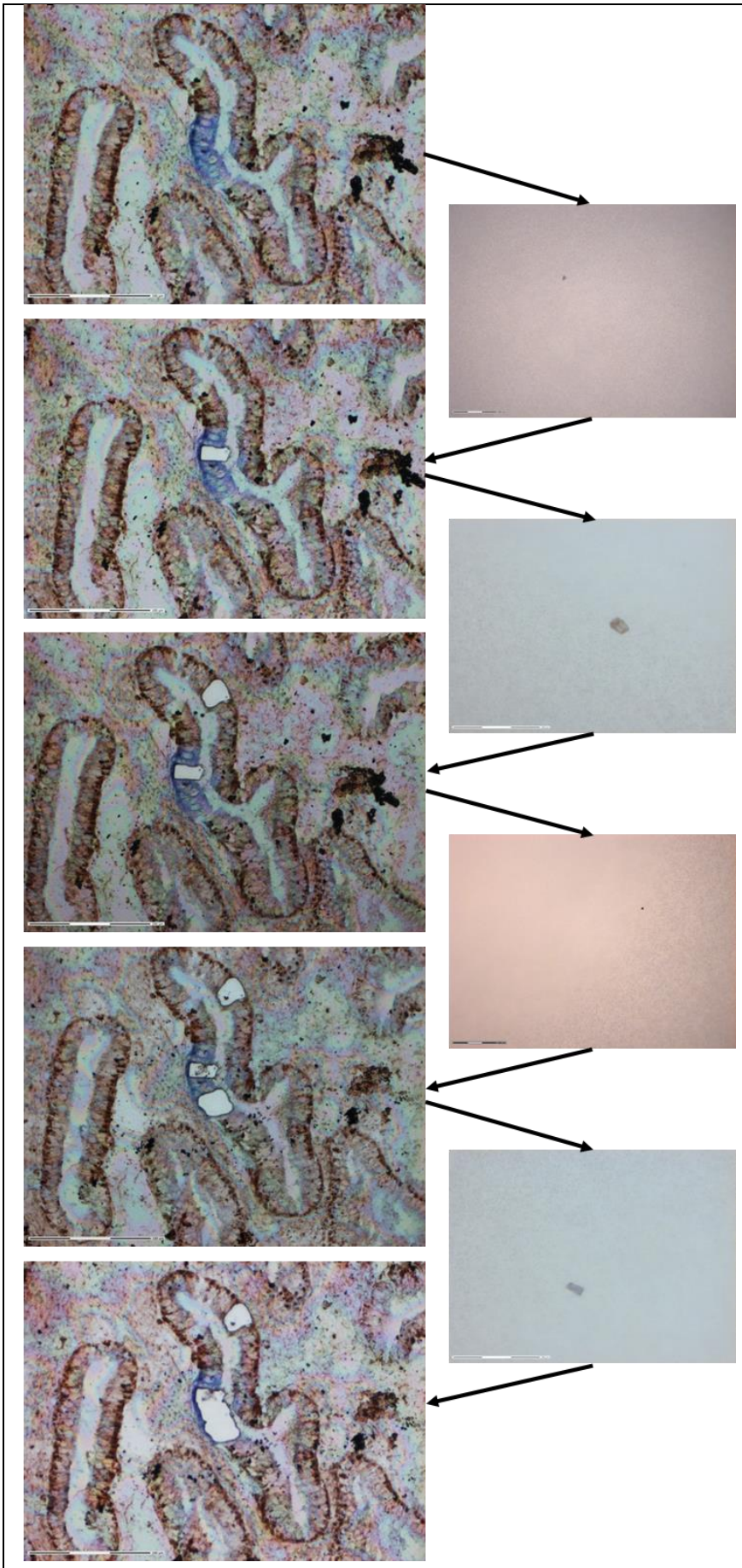


Figure 5.4 – Representative micrographs showing multiple CCO-deficient (blue) cells and adjacent CCO-proficient (brown) cells isolated from the same endometrial gland using LCM. The single epithelial cells can be seen in the adhesive lid of the PCR tube following removal from the tissue.

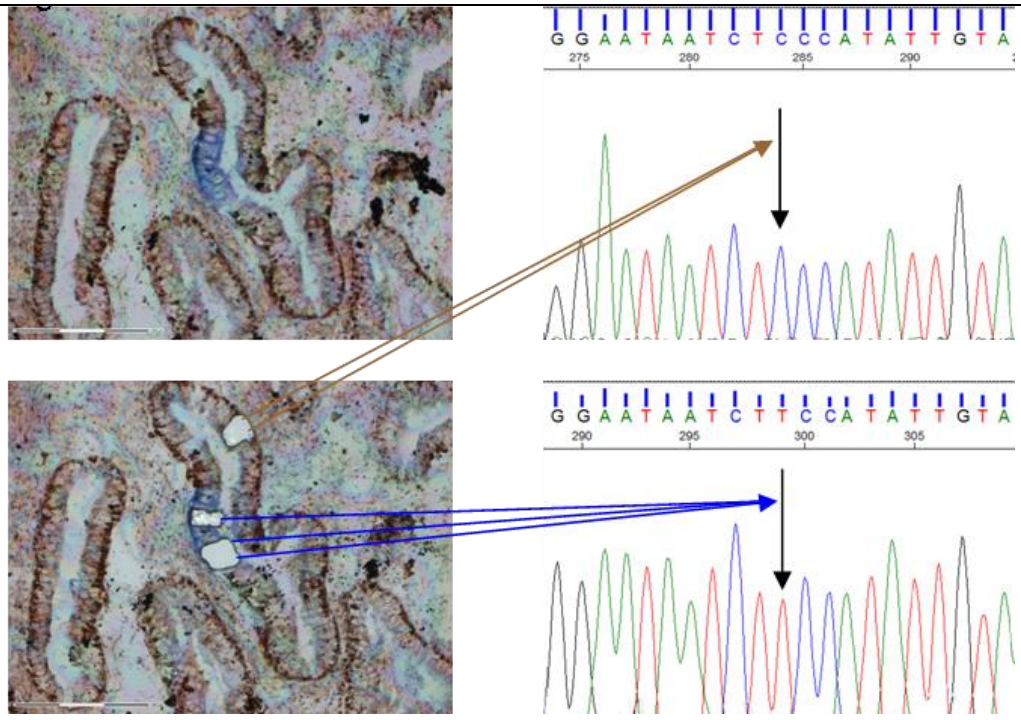
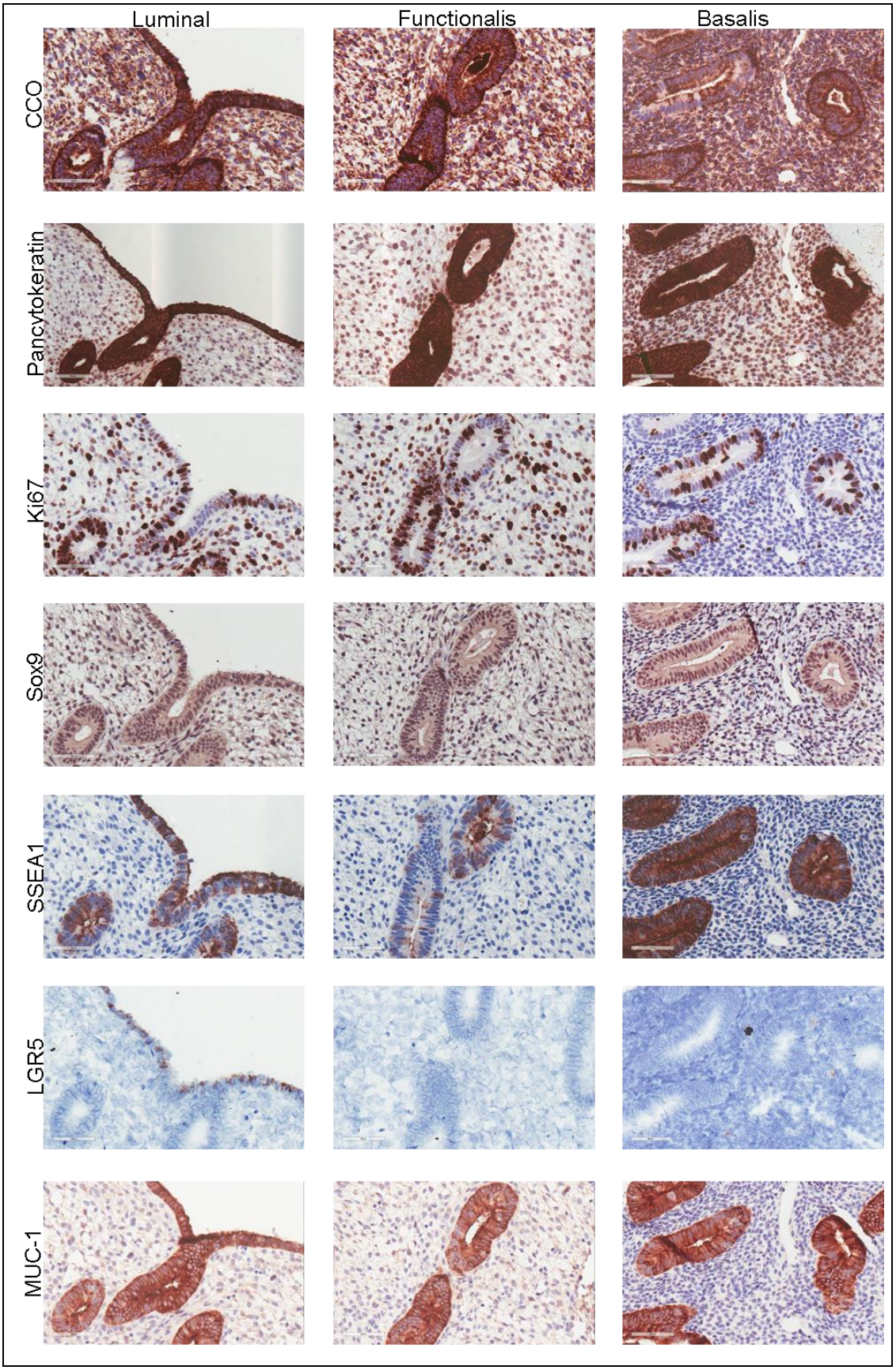


Figure 5.5 – **A Unique mitochondrial mutation confirms the existence of endometrial epithelial stem cells in the endometrium.** Representative images showing all wild type brown CCO-proficient cells with `C` on Sanger sequencing at position 6667 and all blue CCO-deficient cells with `T` on Sanger sequencing at position 6667, demonstrating a clonal expansion.

Endometrial epithelial stem cells are multipotent.

As previously discussed, markers deciphering the different endometrial epithelial cell subpopulations are yet to be confirmed. Therefore, in order to prove that the CCO-deficient epithelial cell progeny contain all endometrial epithelial cell subtypes, we examined the co-expression of markers proposed for the 3 different anatomical regions (luminal, functionalis, and basalis epithelial glands), and of proliferation and epithelium in 2 proliferative and 1

secretory phase endometrial samples. This included *LGR5*, MUC1, BCAM, CK5/6 for LE; SSEA-1, N-Cadherin, and SOX9 for basalis glands; and absence of SSEA-1, *LGR5*, N-Cadherin, SOX9, BCAM, and CK5/6 for the secretory functionalis glands (Figures 5.6, 5.7 and 5.8). The clonal patch containing CCO-deficient epithelial cells contained markers specific to all 3 endometrial regions, suggesting the different cell subtypes comprising the human endometrial glandular compartment has a common ancestral origin, i.e. the existence of a multipotent epithelial stem cell.


Luminal
Functionalis
Basalis
CCO
Pancytokeratin
Ki67
Sox9
SSEA1
LGR5
MUC-1

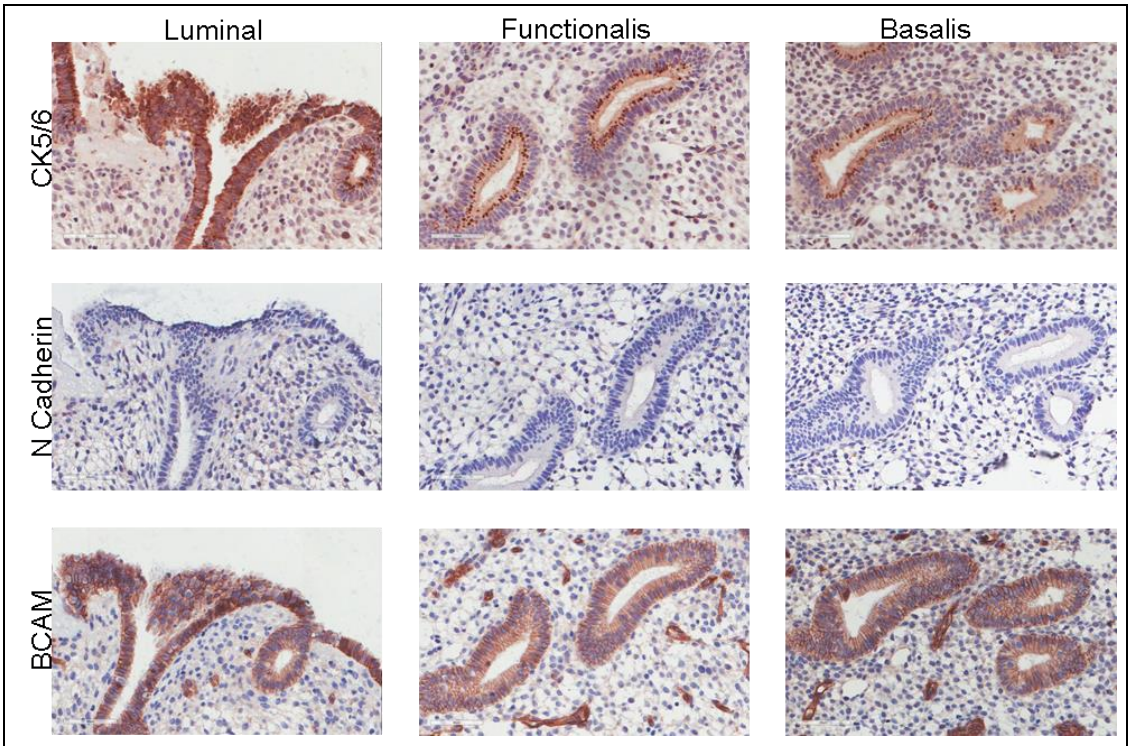
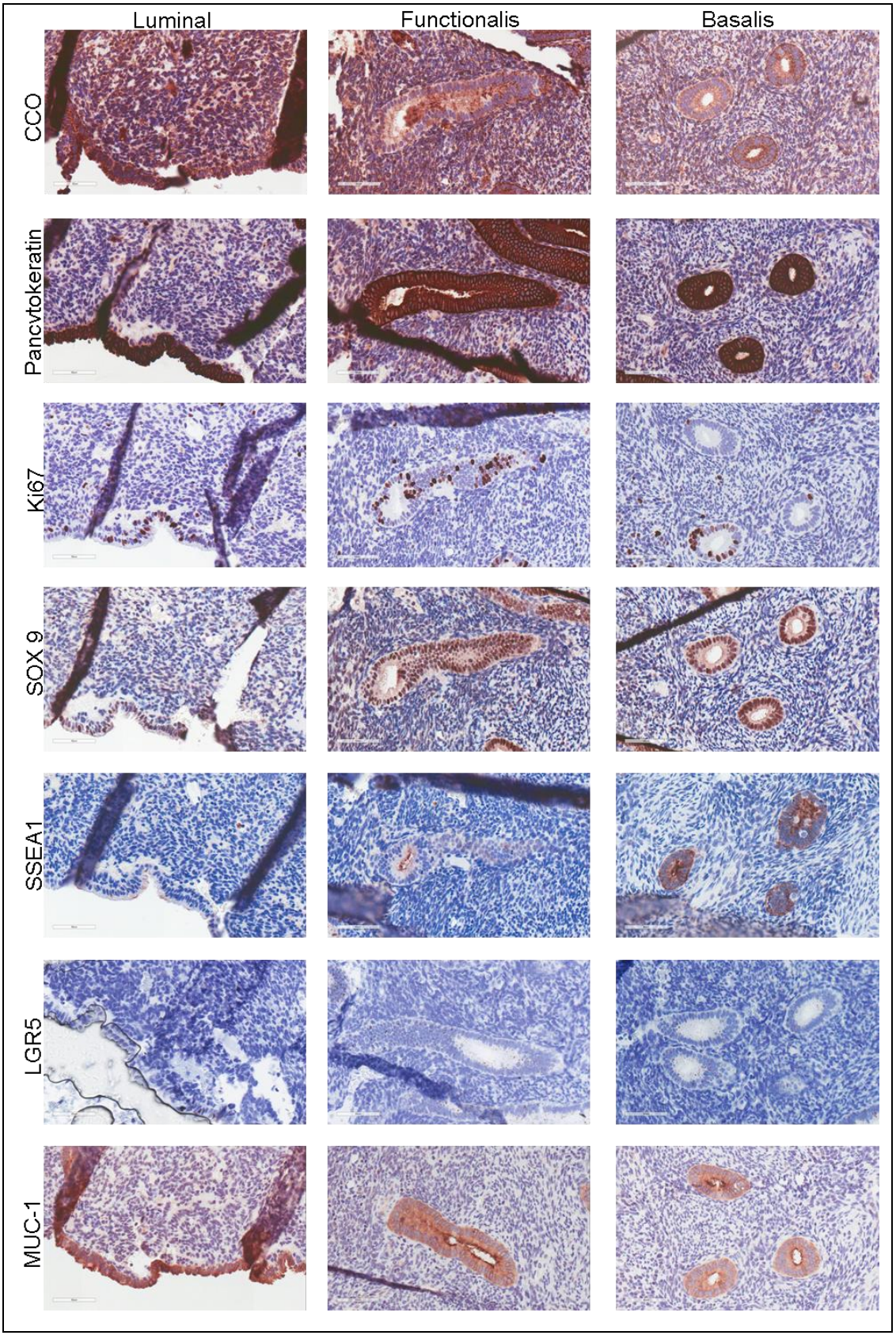


Figure 5.6 - **Endometrial epithelial stem cells are multipotent.** Representative micrographs of consecutive sections of full thickness endometrium depicting CCO-deficient epithelial cells contain markers in all 3 endometrial regions. (All images x400 and scale bars = 10 µm).


Luminal
Functionalis
Basalis
CCO
Pancytokeratin
Ki67
SOX 9
SSEA1
LGR5
MUC-1

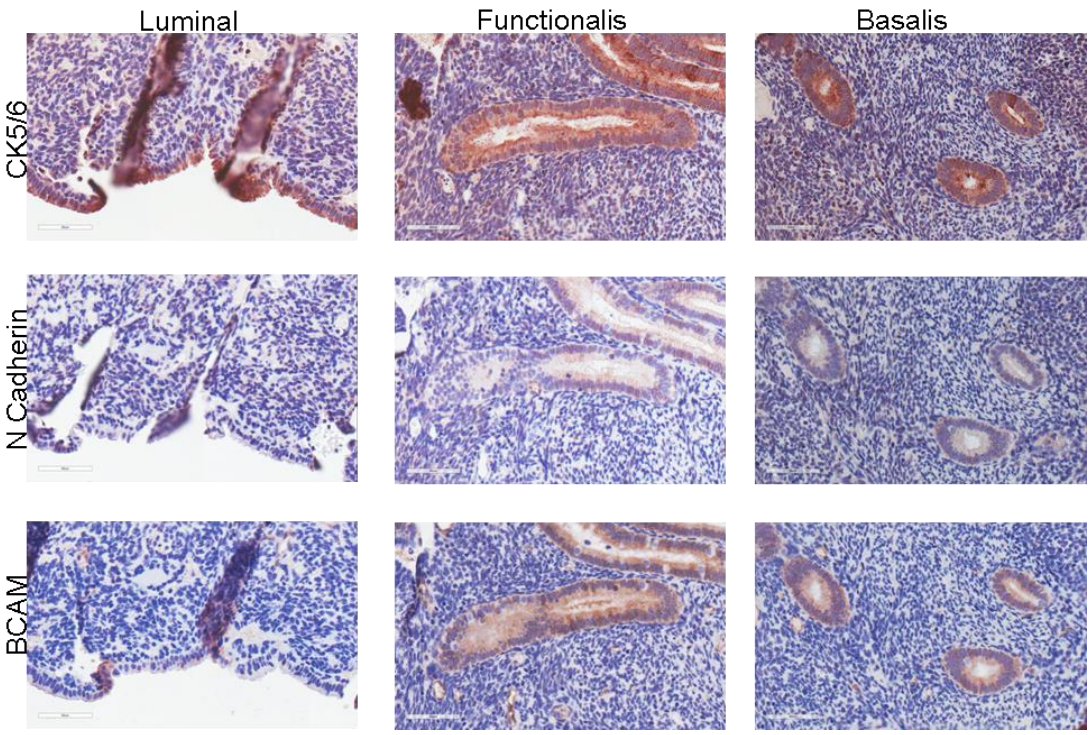
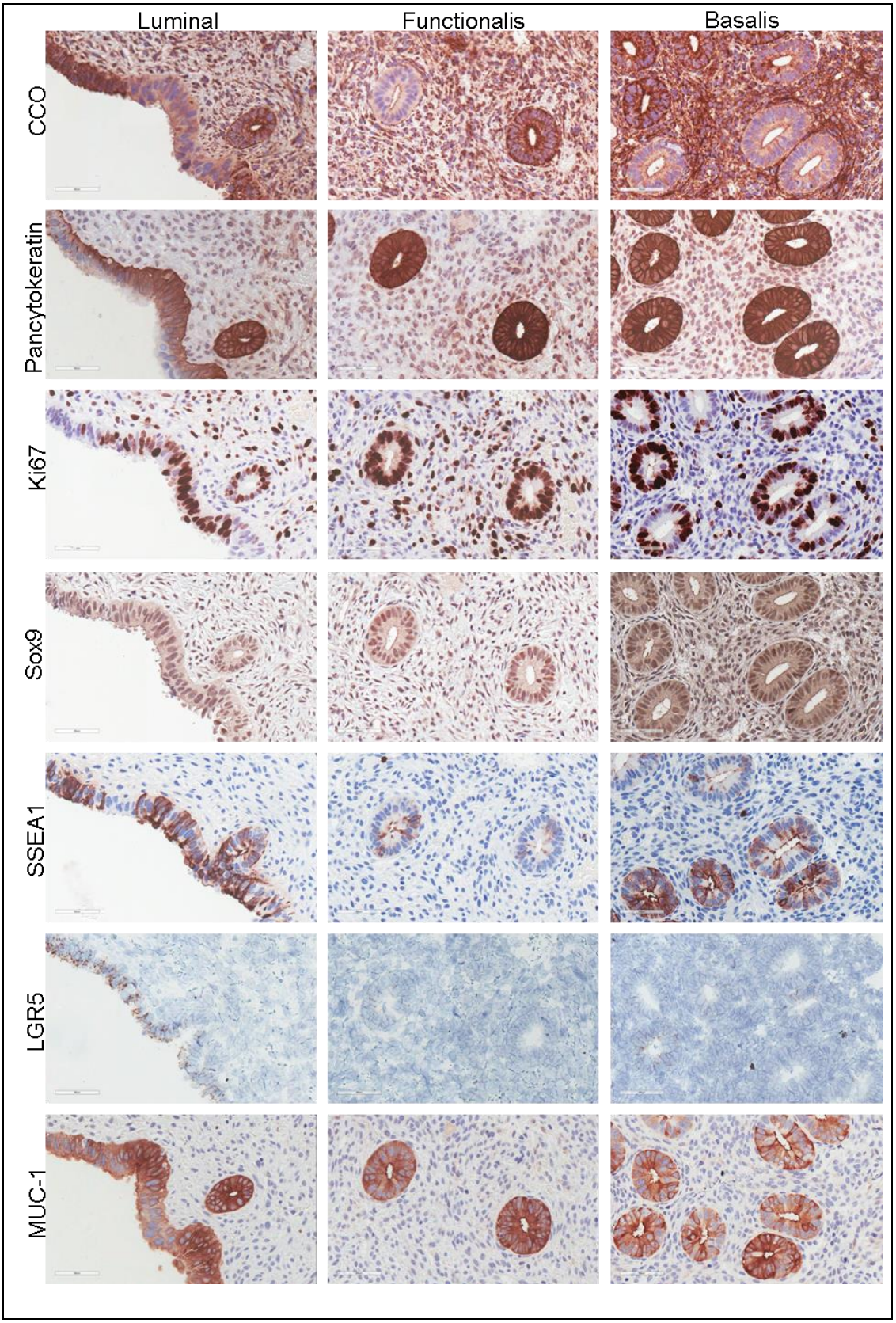


Figure 5.7 - **Endometrial epithelial stem cells are multipotent.** Representative micrographs of the consecutive sections of full thickness endometrium depicting CCO-deficient epithelial cells contain markers in all 3 endometrial regions (All images x400 and scale bars = 10 µm).


Luminal
Functionalis
Basalis
CCO
Pancytokeratin
Ki67
Sox9
SSEA1
LGR5
MUC-1

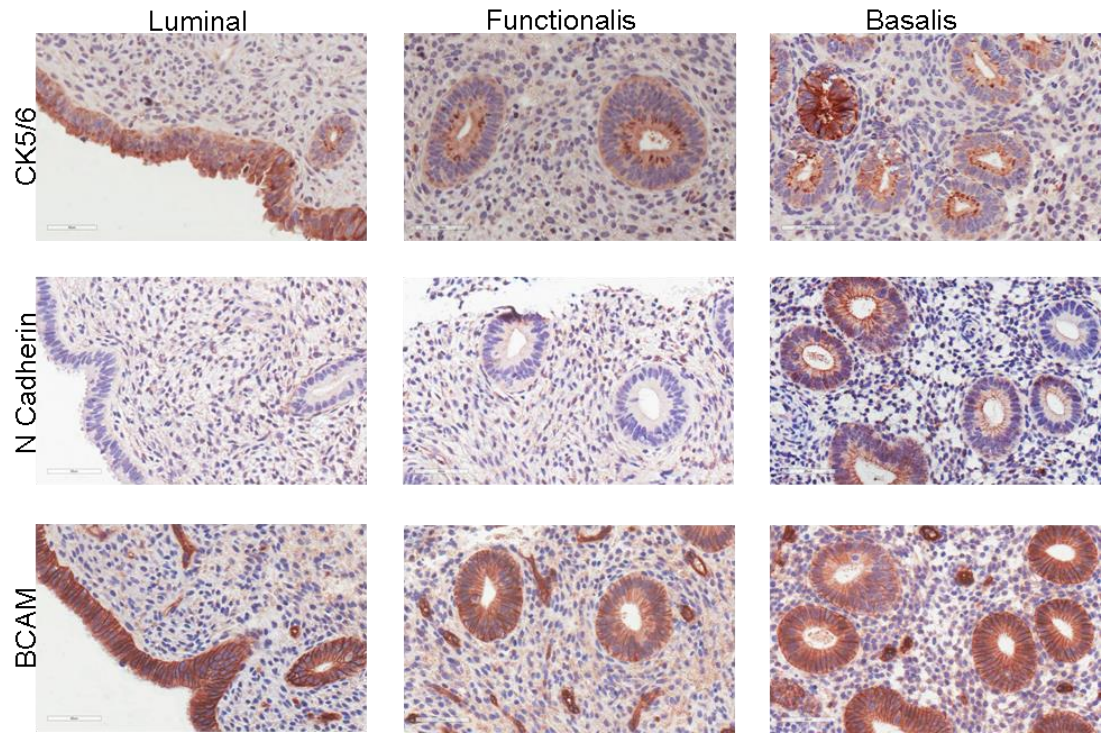


Figure 5.8 - **Endometrial epithelial stem cells are multipotent.** Representative micrographs of consecutive sections of full thickness endometrium depicting CCO-deficient epithelial cells contain markers in all 3 endometrial regions (All images x400 and scale bars = 10 µm).

A 3D-model of endometrial epithelium incorporating CCO negative clonal patches of full thickness endometrium suggests the basalis glands to be the location of the human endometrial epithelial stem cell niche.

The model of CCO staining clearly demonstrates an abundance of mutated glands residing in the basalis glands, which appear to communicate with the functionalis/luminal CCO-deficient epithelial areas in the premenopausal secretory phase endometrial sample (Figure 5.9). Since the functionalis/luminal layers are lost with the menstrual shedding, the clonal patch can be assumed to have arisen from the stem cells located in the basalis glands.

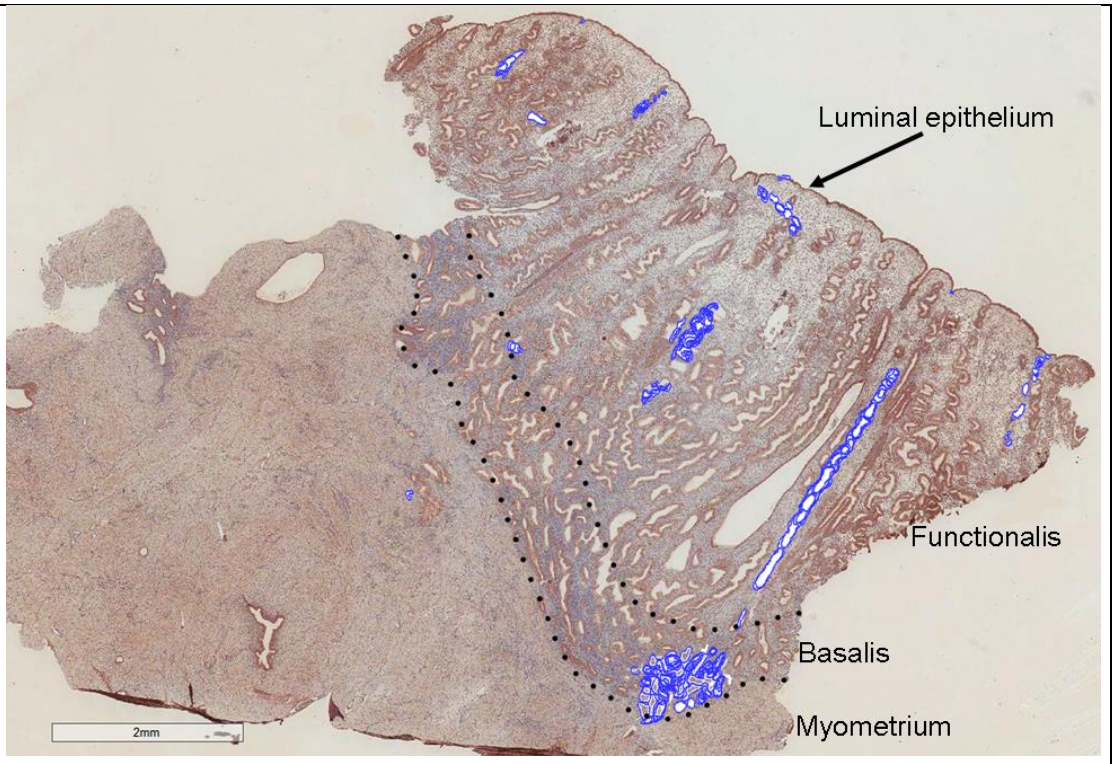


Figure 5.9 – **The extent of the CCO-deficient clonal patches suggests the basalis glands to be the location of the human endometrial epithelial stem cell niche.** Representative image showing the CCO-deficient epithelial cells that occurred across the 100 sections, superimposed onto one section (scale bar = 2 mm).

5.4 **Discussion**

We identified and traced a clonal mtDNA CCO mutation in single cells of human endometrial epithelium, thereby confirming the existence of an endometrial epithelial stem cell for the first time. The observation of partially mutated glands demonstrated the polyclonal nature of some endometrial glands (i.e. more than one stem cell contributes to the gland). CCO-deficient clonal patches also contained cells of all epithelial regions of the endometrium, demonstrating their common origin from a multipotent epithelial stem cell. 3D reconstruction of CCO-deficient patches suggested the epithelial stem cell niche to be located in the basalis region.

CCO–deficient patches appeared in basalis glands of a 25 year old patient, relatively earlier than the reported age when mutations were observed in other mucosal tissue, such as the colon (Greaves, Preston et al. 2006). Previous authors have suggested these patches to be a rare occurrence before the age of 40 years in other mucosal organs. However, this assumption may be at least partly due to the lack of access to human samples from those organs, before that age, for study, rather than a true lack of CCO-deficiency. Nevertheless, the younger women in our cohort did have less CCO-deficient clonal patches than the older age groups, and the location of the mutations were in the basalis, but not the functionalis. This may indicate the origin of these clonal patches also to be the basalis. Interestingly, the prevalence of the CCO-deficient clonal patches increased with age only in the pre-menopausal period. During the reproductive years, due to the repetitive regeneration requirement with the monthly menstrual shedding, endometrial epithelial stem cells are likely to be in an active state. This will promote oxidative stress and, together with advancing age, the active stem cells will accumulate mtDNA mutations. After menopause, in the non-regenerating quiescent endometrium, the clonal patch prevalence remained static. This may be explained by the accumulation of mutations relevant to stem cell activation during the pre-menopausal years. This data agrees with previous work tracking methylation patterns to assess stem cell progeny (Kim, Tavare et al. 2005). The authors proposed methylation patterns in the endometrium to follow the hypothesis that methylation increased after menarche to menopause, but were relatively stable thereafter, indicating that the number of epigenetic marks was a reflection of the mitotic activity of endometrial stem/progenitor cells (Kim, Tavare et al. 2005).

The endometrial samples from nulliparous women had a higher number of CCO-deficient clonal patches (either partial/complete) than the multiparous women. It is already known that nulliparous women are at a higher risk of endometrial cancer due to them having a higher number of ovulatory menstrual cycles (likely because the parous women do not have ovulatory cycles whilst being pregnant or when breast feeding) (Britt and Short 2012),

thus, an increased number of cycles of endometrial regeneration. This may not be an easy hypothesis to test in our patient cohort since many of the nulliparous women may not have undergone more cycles of menstrual regeneration, due to their use of many hormonal contraceptives. This, possible confounding, factor (we are unaware of the complete contraceptive history of our donors) means that the effect of regenerative cycles is difficult to interpret in our patient group (Gleicher 2013).

The cells of CCO-deficient patches were confirmed to harbour the same mtDNA mutation, confirming their common origin, and the existence of an epithelial stem cell. The presence of unique mtDNA mutations in all cells of CCO-deficient areas, but their absence in adjacent CCO-proficient areas, in a partially mutated gland, confirms the contribution from more than one stem cell for the regeneration of such glands, therefore, glandular polyclonality.

Previous studies on glandular clonality are contradictory, with one study using X chromosome inactivation suggesting endometrial glands to be monoclonal in origin (Tanaka, Kyo et al. 2003), whilst another study, which employed tracking of methylation patterns to examine cell progeny, concluded endometrial glands to be of polyclonal origin (Kim, Tavare et al. 2005). Both studies utilised a gland extraction method that is not appropriate for the complex endometrial glands, but to the crypts of the colon, with the presumption that endometrial glandular histo-architecture is a single blunt ended tube. Therefore, both studies are likely only to have considered the functionalis glandular portion. Furthermore, they both examined less than 250 glands in total, which is far less than the number examined in our study, which examined intact undisturbed full thickness human endometrium from a large number of patients, and thousands of individual glands. Additionally, it is notable that the large patch size of X-chromosome inactivation could be a potential confounding factor.

Since endometrial epithelial subtypes are yet to be fully characterised, we examined the expression of regional specific markers according to previous publications, and considered data generated in house from our laboratory.

Here, I have demonstrated the LE to express high levels of *LGR5* gene, BCAM, SSEA-1, SOX9 (Tempest, Baker et al. 2018), CK5/6 (Maclean A 2017), and MUC1 (Hild-Petito, Fazleabas et al. 1996, DeLoia, Krasnow et al. 1998, Dharmaraj, Chapela et al. 2014); the basalis epithelial markers utilised were SSEA-1, SOX9 (Valentijn, Palial et al. 2013), and N-cadherin (Nguyen, Xiao et al. 2017). Ki67 and pancytokeratin were used to confirm that the CCO deficient clonal patches maintained normal proliferation levels, and markers of an epithelial cell. The CCO-deficient clonal patch contained cells stained for all of these markers, as well as cells that do not express LE and basalis markers. Therefore, clonal patches containing cells with characteristic markers of all endometrial regions is indicative of the multipotency of the ancestral epithelial stem cell, capable of contributing to all cells of the whole endometrial epithelial compartment. Finally the 3D gland reconstruction confirms the basalis layer to be the location of at least one epithelial stem cell niche of the human endometrium. We have demonstrated the existence of a basal glandular CCO-deficient clonal patch, extending to the functionalis gland in premenopausal endometrium, thus, our data provides evidence for the existence of an epithelial stem cell niche in the basalis, agreeing with the current dogma. However, we cannot rule out further endometrial epithelial niches, since, even with this model of the secretory phase endometrium, we can see CCO-deficient clonal patches in the LE and functionalis glands that do not appear to communicate directly with a clonal population in the basalis region. Furthermore, this method does not allow visualisation of dynamic spread of the mutation within the model, thus, we cannot comment on if the mutation has arisen from the basalis or descended from the LE. These aspects require further investigation with alternative methods in the future.

Although the existence of an epithelial stem cell in the human endometrium has been postulated for a long time, this is the first direct *in vivo* confirmation of the existence of a human adult endometrial epithelial stem cell. We, for the first time, demonstrated that the human endometrium harbours unique mtDNA mutations, proving the existence of endometrial epithelial stem cells, and that endometrial glandular regeneration is polyclonal. Non-pathogenic

mtDNA mutations present in single CCO-deficient (blue) cells that are not present in single adjacent CCO-proficient (brown) cells indicate that long lived clonal populations exist in the endometrium. Although the method only allows for a single snapshot in time, the technique does demonstrate the two major aspects of stemness: multipotency and self-renewal.

This method could be utilised in the future to investigate the clonality of endometrial hyperplasia and endometrial cancer and, therefore, to examine the role of ASCs in endometrial carcinogenesis, and to discover if stem cells play a role in the pathogenesis of endometriosis or if ectopic lesions are derived from the eutopic endometrium.

Chapter 6. General Discussion

Overview of research findings

During the reproductive years of a woman's life, the human endometrium is one of the most regenerative organs in humans. The endometrium, secondary to its regenerative capacity, was always thought to have a stem cell basis and, subsequently, from the initial studies that identified endometrial cells with some stem cell activity, endometrial stromal stem cells have been thoroughly researched, albeit predominantly, using *in vitro* methods. However, up until now, the existence of endometrial epithelial stem cells has not been conclusively proven. The main difficulty may have been the incompatibility of most *in vitro* analyses for the human endometrial epithelial cells, which are challenging to culture in the laboratory. The work presented in this thesis utilises a method that has not previously been employed in the human endometrium (*in vivo* lineage tracing) to confirm the existence of endometrial epithelial stem cells, as well as illuminating the location of expression, proposing a role for *LGR5* (a presumed universal epithelial stem cell marker) in the endometrial epithelial compartment and, finally, the previously unknown, complex three dimensional (3D) histoarchitecture of the endometrial glands.

Leucine-rich repeat-containing G-protein-coupled receptor-5 emphases future research efforts to define and characterise the endometrial luminal epithelium.

Chapter 3 documents the first comprehensive study employing the gold standard method (*in situ* hybridisation (ISH)) in order to examine the cellular location of *LGR5* expression in full thickness normal human endometrium. The results of this study were unanticipated. Instead of depicting a small number of endometrial epithelial cells in the gland bases (the proposed endometrial epithelial stem cell location) (Valentijn, Palial et al. 2013, Gargett, Schwab et al. 2016), the strongest *LGR5* expression were seen in the endometrial luminal epithelium (LE). The previously proposed endometrial epithelial stem cell niche, the basalis, also contained cells

expressing *LGR5,* but the expression was weaker than the LE. Previous studies have highlighted the need for the LE to be scrutinised as a separate entity to the functionalis epithelium (Evans, Martinez-Conejero et al. 2014), but despite this, the whole of the glandular epithelium (LE, functionalis, and basalis epithelium together) is regularly discussed in many manuscripts. The LE is, obviously, the first point of contact for an embryo that attaches, invades, and implants in the window of implantation in the secretory phase of the cycle. Endometrium is thickest at this time point (up to 16 mm) and, therefore, LE, in cellular terms, exists at a relatively distant location from the basalis. Due to the surprising finding of high *LGR5* expression in LE, we speculated that the LE could, in fact, regenerate itself, with its own pool of progenitor cells. It is known that other epithelial tissues such as the skin, and the intestine (Barker 2014), react to external assaults on a daily basis (mechanical friction or infection), continually replacing cells that are lost. A similar daily cellular loss is likely to be happening at the endometrial surface. The LE (exposed to the uterine cavity and external environment) requiring its daily maintenance by locally positioned cells with progenitor ability. Supporting this hypothesis, rapid *Lgr5*$^{+}$ epithelial cell proliferation can be observed in many other organs upon tissue damage (Ng, Tan et al. 2014, Beumer and Clevers 2016). We merged the *LGR5* data generated with the new hypothesis and proposed Figure 3.9, illustrating that it would be possible that the human endometrium could have more than one epithelial stem/progenitor cell pool. The persistent expression of the progenitor cell markers SOX9 and SSEA-1 in the LE, with concomitant high *LGR5* expression, corroborate further with the above hypothesis (Barker and Clevers 2010, Valentijn, Palial et al. 2013).

A hormonal regulation of *LGR5* expression was observed, examining samples of endometrium taken across the menstrual cycle (oestrogen dominant proliferative phase, and progesterone dominant secretory phase) from healthy women, not on hormonal therapy, and from women who are on progestogens; by quantitative polymerase chain reaction (qPCR) and ISH, and also through *in vitro* experiments.

If *LGR5* was a universal epithelial stem cell marker, one would postulate that the expression be limited to a relatively small number of epithelial cells in a stem cell niche. If they mark the stem/progenitor cells responsible for regeneration of the endometrial functionalis, their numbers may be expected to change at the time of regeneration, or if they mark the more primitive quiescent stem cells producing the progenitor or transient amplifiers (TAs), they would be static throughout the menstrual cycle. This being said, the endometrium is unique in its regenerative ability and its exquisite response to hormones, this may result in a different pattern of *LGR5* expression in the endometrium when compared with other regenerative epithelial tissues.

Future work examining the functional properties of different endometrial epithelial cell subpopulations that are isolated from the three anatomical regions within the human endometrium (which express either high, low, or absent LGR5, SOX9 and SSEA-1), is warranted. Identifying the exact ligand for LGR5 receptor in the human endometrium may also shed light on the functional regulation of these epithelial cells.

The newly found complex 3D Architecture of endometrial glands highlights a potential mechanism for the observed endometrial epithelium self-preservation.

To date, it has been assumed that the endometrial glandular architecture is similar to the intestinal crypt, with blind ending single tubes. These were thought to commence at the LE, and were to extend through the functionalis layer, to end as the basalis glands at the basalis-myometrial junction. Although there are a lot of similarities between the endometrial epithelium and the intestinal crypts, striking differences are also present; for example, the monthly shedding of the functionalis. The endometrial glands have a much more complex coiling architecture, which changes progressively through the menstrual cycle, under the influence of oestrogen and progesterone. The current consensus of considering the endometrial glands as blind ending tubes has ignored some previous studies, suggesting an alternative architecture of the endometrial glands (Ferenczy and Richart 1973, Cooke, Spencer et al. 2013); although only two dimensional (2D)

tissue sections were considered. The work undertaken, as presented in Chapter 4, proposes a new glandular architectural arrangement (Figure 4.9), after developing 3D models from 3 full thickness tissue samples of human endometrium. This newly constructed endometrial glandular layout demonstrated non-branching vertical parallel functionalis glands, and branching, intricate basalis networks running horizontal to the myometrium. The purpose of this complex glandular architecture generates the hypothesis that it is required for efficient endometrial regeneration. For example, the current presumed single tubular architecture of the glands may be an ineffective way of ensuring regeneration, since, if a basal-end of the gland is lost by iatrogenic curettage, it will be detrimental to endometrial regeneration in that area, and will result in endometrial scarring. However, with root-like, complex basalis glands running at a horizontal course, a damaged basalis glandular zone can be efficiently repaired by the adjacent, stem-cell rich, basalis glands. The basalis glands are more likely to be preserved, covering the myometrial surface, to assist in scar-less endometrial self-renewal, not just on a monthly basis, but also following parturition, iatrogenic destruction (after surgical ablation), and curettage. It is tempting to assume that relying on the previously proposed architecture may not be the most efficacious arrangement, as the basalis glandular ends could potentially be easily lost/artificially removed during one of the above situations, leading to the loss of the regenerative ability of the endometrium in the near vicinity. The complex root-like stolen configuration provides the ideal rapid replenishment of the stem cell niche for the entire basalis glandular element.

The work presented in this chapter commences a novel avenue of endometrial 3D architectural work, and needs to be expanded to study normal endometrium, i.e. the postmenopausal (PM) endometrium, many human endometrial pathologies such as endometrial hyperplasia, and endometrial cancer. Further work is warranted to examine if the normal 3D architecture is altered in common recurrent endometrial pathologies that interfere with the normal function of human embryo-implantation, such as subfertility and recurrent miscarriage.

The existence of endometrial epithelial stem cells instigates endometrial pathology research.

Chapter 5 provides confirmation of the existence of endometrial epithelial stem cells. Using the gold standard method (*in vivo* cell lineage tracing), a unique mitochondrial DNA (mtDNA) cytochrome C oxidase (CCO) mutation in single cells from a clonal population of human endometrial epithelium, was identified; confirming the existence of endometrial epithelial stem cells for the first time. This work further demonstrated partially mutated glands, producing evidence for the polyclonal nature of at least some endometrial glands (i.e. more than one stem cell contributes to the gland). The CCO-deficient clonal patches incorporated cells of the LE, functionalis, and basalis epithelium, demonstrating their common origin. 3D reconstruction of CCO-deficient patches suggested the location of at least one epithelial stem cell niche to be in the basalis region. This method of natural lineage tracing allows only for a single snapshot in time, but, the technique does demonstrate the two major aspects of stemness: multipotency and self-renewal.

The finding that endometrial epithelial stem cells exist is only the start of this work, in order to extend our current understanding of the endometrial regeneration, and to eventually help women suffering with endometrial proliferative disorders, more work is to be done investigating the clonal nature of endometrial hyperplasia and endometrial cancer; and also in the benign, chronic condition (currently without adequate treatment) endometriosis. Since stem cells are likely to be abnormal in these conditions, (thus, regenerating an abnormal endometrium each month), treating stem cell abnormalities will potentially cure the disease. Understanding how endometriosis develops, if it is originating from the eutopic endometrium, will allow us to classify, treat, and potentially cure women suffering with this condition. Furthermore, by determining if stem cells play a role in endometrial cancer, and knowing their exact location, will aid in the development of targeted treatment options.

Strengths and limitations of this research

Immunoscoring of IHC and ISH analysis was open to observer subjectivity. We controlled for this bias by at least two observers scoring the sections independently, in a set of randomly selected samples (assessing the inter-observer reproducibility), and by revisiting the samples and scoring them again personally (assessing intra-observer reproducibility). The *LGR5* ISH scores were analysed using a scoring system that was optimised, and recommended, by the manufacturers of the ISH kit (Advanced Cell Diagnostics, Hayward, CA), and by previous publications (Baker, Graham et al. 2015).

Although a larger sample set is always preferable when undertaking experiments, to account for patient related heterogeneity, there are obvious limitations to the numbers that can be realistically included. For example, the challenges encountered included: collecting appropriately dated, matched, eutopic and Fallopian tube samples from women who are not on hormonal treatment, without known endometrial pathologies for paraffin embedding, frozen sectioning, and extraction of RNA; Identifying full thickness endometrial blocks that can provide 100 consecutive sections. Appropriate numbers of samples were collected in order to produce data that can be interrogated with statistical testing for significance, and to provide conclusions. The methodology required to produce the data presented in the thesis was time consuming to master (e.g. single cell DNA extraction and sequencing), and to perform (3D reconstruction). Producing 3D models with 100 consecutive sections from a PM sample, stained with CCO would have been ideal, unfortunately, the technical challenges with the collected PM samples, and time pressures, precluded that. Co-localising the known epithelial region specific markers, such as SSEA-1, SOX9, N-cadherin, *LGR5* etc, with CCO staining, using alternative methods such as IF, would further confirm the data presented using IHC in the consecutive tissue sections.

Conclusion, impact and future direction

The work described in this thesis presents three novel findings in the area of endometrial research that represent `stepping stones` in order to develop

new ways of thinking about research in the field of endometrial epithelial regeneration. They encourage development of new theories and hypothesis regarding the different epithelial compartments, the 3D architecture of the glands, and the stem cell makeup of the endometrium; for the enhancement of our current understanding of the human endometrium. *LGR5* expression in the human endometrial epithelium has presented a new conundrum regarding the location of the stem cell niche in the human endometrial epithelium. The 3D reconstruction of the endometrial epithelium has shown that, previously proposed, non-branching, vertical and parallel glandular architecture, is limited only to the functionalis glands but intricate, branching, horizontal basalis glands, form a previously unknown root-like 'stolen' architecture, which instigates many further queries regarding the glandular architectural changes in various pathologies. The direct confirmation of the existence of endometrial epithelial stem cells, utilising non-pathogenic mtDNA mutations and lineage tracing for the first time, will re-ignite the race to find markers to further examine them in the future. For those reasons, the work presented in here is envisaged to pave the way to enhance the field of endometrial and gynaecology research in general in the future.

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ORIGINAL ARTICLE *Gynaecology*

Does human endometrial LGR5 gene expression suggest the existence of another hormonally regulated epithelial stem cell niche?

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STUDY QUESTION: Is human endometrial leucine-rich repeat-containing G-protein-coupled receptor 5 *(LGR5)* gene expression limited to the postulated epithelial stem cell niche, stratum basalis glands, and is it hormonally regulated?

SUMMARY ANSWER: *LGR5* expressing cells are not limited to the postulated stem cell niche but *LGR5* expression is hormonally regulated.

WHAT IS KNOWN ALREADY: The human endometrium is a highly regenerative tissue; however, endometrial epithelial stem cell markers are yet to be confirmed. LGR5 is a marker of stem cells in various epithelia.

STUDY DESIGN, SIZE, DURATION: The study was conducted at a University Research Institute. Endometrial samples from 50 healthy women undergoing benign gynaecological surgery with no endometrial pathology at the Liverpool Women's hospital were included and analysed in the following six sub-categories; proliferative, secretory phases of menstrual cycle, postmenopausal, those using oral and local progestagens and samples for *in vitro* explant culture.

PARTICIPANTS/MATERIALS, SETTING, METHODS: In this study, we used the gold standard method, *in situ* hybridisation (ISH) along with qPCR and a systems biology approach to study the location of *LGR5* gene expression in full thickness human endometrium and Fallopian tubes. The progesterone regulation of endometrial *LGR5* was examined *in vivo* and in short-term cultured endometrial tissue explants *in vitro*. *LGR5* expression was correlated with epithelial proliferation (Ki67), and expression of previously reported epithelia progenitor markers (SOX9 and SSEA-1) immunohistochemistry (IHC).

MAIN RESULTS AND THE ROLE OF CHANCE: *LGR5* gene expression was significantly higher in the endometrial luminal epithelium than in all other epithelial compartments in the healthy human endometrium, including the endometrial stratum basalis ($P < 0.05$). The strongest SSEA-1 and SOX9 staining was observed in the stratum basalis glands, but the general trend of SOX9 and SSEA-1 expression followed the same cyclical pattern of expression as *LGR5*. Stratum functionalis epithelial Ki67-LI and *LGR5* expression levels correlated significantly ($r = 0.74$, $P = 0.01$), however, they did not correlate in luminal and stratum basalis epithelium ($r = 0.5$ and 0.13, respectively). Endometrial *LGR5* demonstrates a dynamic spatiotemporal expression pattern, suggesting hormonal regulation. Oral and local progestogens significantly reduced endometrial *LGR5* mRNA levels compared with women not on hormonal treatment ($P < 0.01$). Our data were in agreement with *in silico* analysis of published endometrial microarrays.

LARGE SCALE DATA: We did not generate our own large scale data but interrogated publically available large scale data sets.

LIMITATIONS, REASONS FOR CAUTION: In the absence of reliable antibodies for human LGR5 protein and validated lineage markers for the various epithelial populations that potentially exist within the endometrium, our study does not formally characterise or examine the functional ability of the resident $LGR5^+$ cells as multipotent.

WIDER IMPLICATIONS OF THE FINDINGS: These data will facilitate future lineage tracing studies in the human endometrial epithelium; to identify the location of stem cells and further complement the *in vitro* functional studies, to confirm if the *LGR5* expressing epithelial cells indeed represent the epithelial stem cell population.

STUDY FUNDING/COMPETING INTEREST(S): This work was supported by funding from the Wellbeing of Women project grant (RTF510) and Cancer Research UK (A14895). None of the authors have any conflicts of interest to disclose.

Key words: endometrial epithelial stem cells / leucine-rich repeat-containing G-protein-coupled receptor 5 / progesterone regulation / in situ hybridisation / stem cell niche / fallopian tube

Introduction

The human endometrium is a highly regenerative tissue, which undergoes over 400 cycles of menstrual shedding and re-growth in a woman's life time. It is composed of two functionally distinct layers, the superficial stratum functionalis and the deeper stratum basalis.

The stratum functionalis is completely shed with menstruation and fully regenerated within 2 weeks, up to a thickness of 16 mm (Fleischer, 1999). This impressive regeneration implies that a stem cell population may reside within the endometrial glands. The location of stem/progenitor cells of the endometrium is postulated to be within the stratum basalis, which remains after the menstrual shedding of the stratum functionalis (Prianishnikov, 1978; Gargett *et al.*, 2016).

Of the two main endometrial specific cell types, the mesenchymal stem/progenitor cells that give rise to stromal cells are well described and studied (Gargett *et al.*, 2016). However, the evidence for an endometrial epithelial stem cell population is sparse. Previous work suggests that SSEA-1 and SOX9 expressing epithelial cell subpopulations have some ability to generate gland-like structures *in vitro* (Valentijn *et al.*, 2013; Turco *et al.*, 2017), but as yet there are no other epithelial markers with the location or functional characterisation suggestive of stem cell specificity described in the endometrium.

Leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) is a transmembrane receptor (Barker *et al.*, 2007) which belongs to a family of glycoprotein hormone receptors (Sun *et al.*, 2009). LGR5 is a marker of stem cells in various epithelia such as the intestinal mucosa (Schuijers and Clevers, 2012), gastric mucosa (Barker *et al.*, 2010), hair follicles (Jaks *et al.*, 2008) and kidneys (Barker *et al.*, 2012). In mammary epithelium *Lgr5*+ cells contribute to both luminal and basal epithelia (de Visser *et al.*, 2012) and are essential to reconstituting mammary glands from single cells (Plaks *et al.*, 2013). In the intestine, *LGR5* was shown to be a Wnt target gene, regulating epithelial regeneration with a restricted expression (visualised by *in situ* hybridisation (ISH)) in the intestinal crypt base (Barker *et al.*, 2007; Schuijers and Clevers, 2012). These basal crypt cells, were previously proposed to be an adult intestinal stem cell population, but their formal functional confirmation awaited the discovery of a specific marker (Leushacke and Barker, 2012). Subsequent work on *Lgr5*+ cells from the intestine, using *in vivo* lineage tracing and a heritable-inducible lacZ reporter gene, showed that *Lgr5*+ cells are long-lived, multipotent stem cells (Gerbe *et al.*, 2011), and a single $Lgr5^{+}$ stem cell can form organoids with a gut-like architecture containing all epithelial cell types (Schuijers and Clevers, 2012).

LGR5 is expressed in the female reproductive organs. *Lgr5* marks stem/progenitor cells of the rodent ovary and the oviduct (Flesken-Nikitin *et al.*, 2013; Ng *et al.*, 2014) where it is critical for the maintenance of a functional corpus luteum and therefore, for successful pregnancy (Sun *et al.*, 2014). In immature and in ovarian hormone deprived mice, *Lgr5* is highly expressed in the single layer of epithelia lining the uterine cavity and progesterone treatment down-regulated *Lgr5*, suggesting an ovarian hormonal regulation (Sun *et al.*, 2009; Boretto *et al.*, 2017). However, mice do not menstruate, their oestrous cycle is characterised by complete reabsorption of the endometrial lining and therefore their epithelial regeneration pattern is proposed to be distinct from women (Gargett *et al.*, 2016). In the human ovary and distal Fallopian tube (fimbriae), *LGR5* expression was confirmed by quantitative reverse transcription PCR (qRT-PCR) (Ng *et al.*, 2014), with constitutive *LGR5* mRNA expression reported in healthy human endometrial epithelium throughout the menstrual cycle (Schuijers and Clevers, 2012; Krusche *et al.*, 2007).

The specificity of the available anti-human-LGR5 antibodies is disputed and in general, the antibody based protein expression data do not correlate with RNA data (Munoz *et al.*, 2012). Thus, ISH is considered as the gold standard to detect *LGR5* expressing cells in a solid tissue (Munoz *et al.*, 2012). Therefore in the human endometrium, antibody based studies need further validation (Cervello *et al.*, 2017; Gil-Sanchis *et al.*, 2013) to confirm the exact *LGR5* expressing cell population and to elucidate the function and regulation of the *LGR5* gene in those cells.

We examined the cellular localisation of *LGR5* in all epithelial compartments of the human endometrium by ISH. As the human Fallopian tube shares the same embryological origin and exists as a continuum with the endometrium, we compared the expression of *LGR5* in the epithelial mucosa of the endometrium with that of the fimbrial end of the Fallopian tube (due to its known stem cell enrichment (Auersperg, 2013)) and *Lgr5* expression (Ng *et al.*, 2014). The hormone regulation of *LGR5* in the endometrium was also studied *in vitro* and *in vivo*. Finally, published microarray datasets were interrogated to confirm *LGR5* expression and its progesterone regulation in the endometrium.

Materials and Methods

Human tissue

Human endometrium and tubal fimbriae was obtained from 50 women undergoing benign gynaecological surgery with no endometrial pathology at the Liverpool Women's hospital (Supplementary Table SI), granted under Local Research Ethics (REC references; 09/H1005/55 and 11/H1005/4). Informed consent was obtained from all patients.

The cycle phase of the endometrium was assigned according to the last menstrual period and histological criteria (Noyes *et al.*, 1975; Dallenbach-Hellweg, 2012). Endometrium and the distal (fimbrial) end of the Fallopian tube samples were divided in to two pieces; one was fixed (≥24 h in 4%

(v/v) buffered formalin) and paraffin-embedded for ISH and immunohistochemical (IHC) staining, and the other immediately placed in to RNA*later* (Sigma, Dorset, UK) for RNA extraction and qRT-PCR. A further six endometrial samples from the proliferative phase of the cycle were collected in reduced serum (1%) Dulbecco's modified Eagle's medium (DMEM)/F12 media for short-term explant culture. ISH and IHC staining for all antibodies was analysed with specific reference to the three different endometrial epithelial compartments, the luminal epithelium (the single layer of cells that forms the luminal surface or lining of the uterine cavity), the stratum functionalis (glands in the upper two-thirds of the endometrium below the luminal epithelium, surrounded by sparse stroma) and the stratum basalis (glands in the lower one-third of the endometrium adjacent to the endo-myometrial junction, surrounded by densely packed stroma) in full-thickness endometrial tissue sections. Sequential sections were stained with pancytokeratin to confirm the assignment of epithelial compartment.

qRT-PCR

Total RNA from tissue samples was extracted using TRIzol Plus RNA Purification System (Life Technologies, Paisley, UK), and quantified by NanoDrop ND-1000 (Thermo Fisher Scientific, Loughborough, UK). Total RNA was reverse transcribed using AMV First Strand cDNA synthesis kit (New England Bio Labs, Hertfordshire, UK) after DNase treatment (DNase I (#M0303), New England Bio Labs, Hertfordshire, UK), using the manufacturer's protocol as previously described (Kamal *et al.*, 2016). cDNA was amplified by qPCR using iTaq SYBR Green supermix (Biorad) with the Biorad connect and the following primers: LGR5 forward (5′-CCTGCTTGACTTT GAGGAAGACC), LGR5 reverse (5′-CCAGCCATCAAGCAGGTGTT CA), GAPDH forward (5′-AATCCCATCACCATCTTCCA) and GAPDH reverse (5′-TGGACTCCACGACGTACTCA). Relative transcript expression was calculated using the ΔΔCT method, normalised to the reference gene GAPDH, using Biorad CFX manager.

ISH

ISH for *LGR5* expression was performed as previously described (Baker *et al.*, 2015) using the RNAscope 2.5 High Definition Brown assay according to the manufacturer's instructions (Advanced Cell Diagnostics, Hayward, CA) as detailed in supplementary methods. RNAscope probes used were *LGR5* (NM_003667.2, region 560–1589, catalogue number 311021), *POLR2A* (positive control probe, NM_000937.4, region 2514–3433, catalogue number 310451) and *dapB* (negative control probe, EF191515, region 414–862, catalogue number 310043) (Supplementary Fig. S1). *LGR5* expression was quantified according to the five-grade scoring system recommended by the manufacturer previously described (Baker *et al.*, 2015) (0 = No staining or less than 1 dot to every 10 cells (40× magnification), 1 = 1–3 dots/cell (visible at 20–40× magnification), 2 = 4–10 dots/cell, very few dot clusters (visible at 20–40× magnification), 3 = >10 dots/cell, less than 10% positive cells have dot clusters (visible at 20× magnification), 4 = >10 dots/cell, more than 10% positive cells have dot clusters (visible at 20× magnification)).

IHC

IHC was performed on sequential tissue sections according to standard protocol as previously described (Valentijn *et al.*, 2013). Primary antibodies (mouse pan-monoclonal anti-cytokeratin (C2562, Sigma-Aldrich, Dorset, UK) at 1:4000, goat polyclonal anti-SOX9 (af3075, R&D Systems, Abingdon,UK) at 1:400, mouse monoclonal anti-Ki67 (NCL-Ki67-MM1, Novocastra, Newcastle, UK) at 1:200, mouse monoclonal anti-SSEA-1 (125601/2, Biolegend, San Diego, CA) at 1:800 dilution) were incubated overnight at 4°C in a humidified chamber. All slides were scanned using an Aperio CS2 scanner (http://www.leicabiosystems.com/digital-pathology/aperio-digital-pathology-slide-scanners/products/aperio-cs2/) and analysed using spectrum, ScanScope®.

Analysis of IHC

Percentage of nuclear Ki67 immuno-positive cells of any intensity was evaluated as the Ki67-labelling index (Ki67-LI) (Al Kushi *et al.*, 2002; Kamal *et al.*, 2016) and the three epithelial compartments were scored separately. SOX9 and SSEA-1 immunostaining was assessed as previously described (Valentijn *et al.*, 2013), and detailed in Supplementary methods.

Explant culture

Endometrial explant cultures were prepared from freshly collected endometrial biopsies and treated with 1 μM medroxyprogesterone acetate (MPA) or ethanol (vehicle control) for 24 h as previously described(Valentijn *et al.*, 2015). Harvested tissue after treatment was washed with PBS, immersed in RNA*later* and frozen for qRT-PCR (Valentijn *et al.*, 2015).

Systems biology

We extended our experimental data by examining all published microarray datasets of normal, premenopausal endometrial samples from women not on hormonal treatments to explore progesterone regulation of the *LGR5* gene in the secretory compared with the proliferative menstrual cycle phase ($n = 65$) (Talbi *et al.*, 2006; Burney *et al.*, 2007; Nguyen *et al.*, 2012; Sigurgeirsson *et al.*, 2017) and in the sorted healthy normal endometrial epithelial side population cells that enrich for the endometrial epithelial stem cell population, against unsorted epithelial cells (Cervello *et al.*, 2010) ($n = 8$/group). The *in silico* methodology using oPPOSUM (http://www.cisreg.ca/oPOSSUM/), Con Tra V3, illumina's BaseSpace Correlation Engine (BSCE; (Kupershmidt *et al.*, 2010) software; https://www.illumina.com/informatics/research/biological-data-interpretation/nextbio.html; Illumina, San Diego, CA, USA) and Ingenuity (IPA) software programmes is detailed in supplementary methods (Supplementary Table II–IV). (Mathew *et al.*, 2016; Broos *et al.*, 2011; Kupershmidt *et al.*, 2010; Cervello *et al.*, 2010; Burney *et al.*, 2007; Talbi *et al.*, 2006; Nguyen *et al.*, 2012; Sigurgeirsson *et al.*, 2017)

Statistical methods

All statistical analyses were performed using GraphPad Prism software (Mann–Whitney *U* and one-way ANOVA was used to assess differences between groups). Spearman rank correlation was used to determine the association between pairs of variables. The criterion for significance was $P \leq 0.05$.

Results

Healthy human premenopausal endometrium demonstrated dynamic spatiotemporal regulation of *LGR5* expression with high *LGR5* expressing cells in the luminal and in the stratum basalis epithelium

Full thickness whole endometrial tissue samples containing all endometrial layers and cell types from the oestrogen dominant, proliferative phase of the cycle showed a trend of higher *LGR5* mRNA expression levels (as measured by qRT-PCR) compared with the samples from the progesterone dominant secretory phase of the menstrual cycle ($P = 0.5$, Fig. 1A). *LGR5* mRNA levels were significantly higher in the stem cell rich distal Fallopian tubes than in the

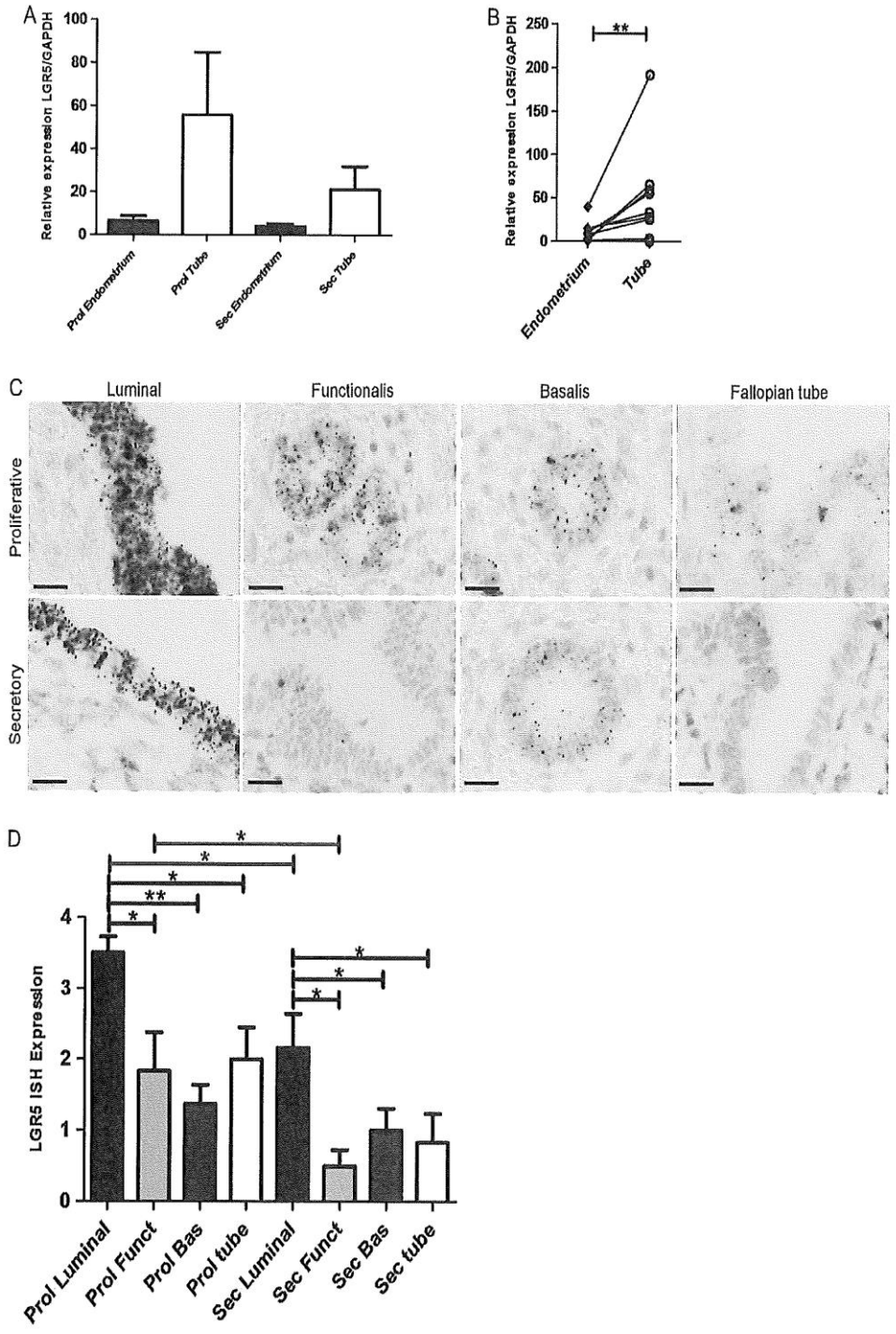

Figure 1 *LGR5* gene expression appears to decrease in the eutopic endometrium and Fallopian tube in the secretory phase of the menstrual cycle. (**A**) The eutopic endometrial samples and fallopian tube express apparently decreased levels of *LGR5* mRNA in the secretory phase of the menstrual cycle when compared with the proliferative phase ($n = 21$). (**B**) Fallopian tube (at any stage of the cycle) demonstrate significantly higher levels of *LGR5* mRNA than matched eutopic endometrium ($P < 0.01$) ($n = 20$). (**C**) Representative *LGR5* ISH images of Fallopian tube and luminal, stratum functionalis and stratum basalis eutopic endometrium in the proliferative and secretory stages of the menstrual cycle (All images ×1000, scale bar = 20 μm, ($n = 15$)). (**D**) Graphical representation of semi-quantitative scoring of *LGR5* ISH In the proliferative stage of the cycle, the luminal epithelium demonstrated significantly higher *LGR5* ISH staining scores than the functionalis ($P < 0.05$), basalis epithelium ($P < 0.01$) and Fallopian tube ($P < 0.02$) as well as the luminal epithelium of the secretory phase ($P < 0.03$); the proliferative functionalis had significantly higher *LGR5* scores than the secretory functionalis ($P < 0.04$); the secretory luminal epithelium showed significantly higher *LGR5* scores than the epithelia of secretory stratum functionalis ($P < 0.03$), secretory stratum basalis ($P < 0.05$) and Fallopian tube ($P < 0.02$) ($n = 7$ per group) (Prol = Proliferative, Sec = Secretory Funct = stratum Functionalis, Bas = stratum Basalis).

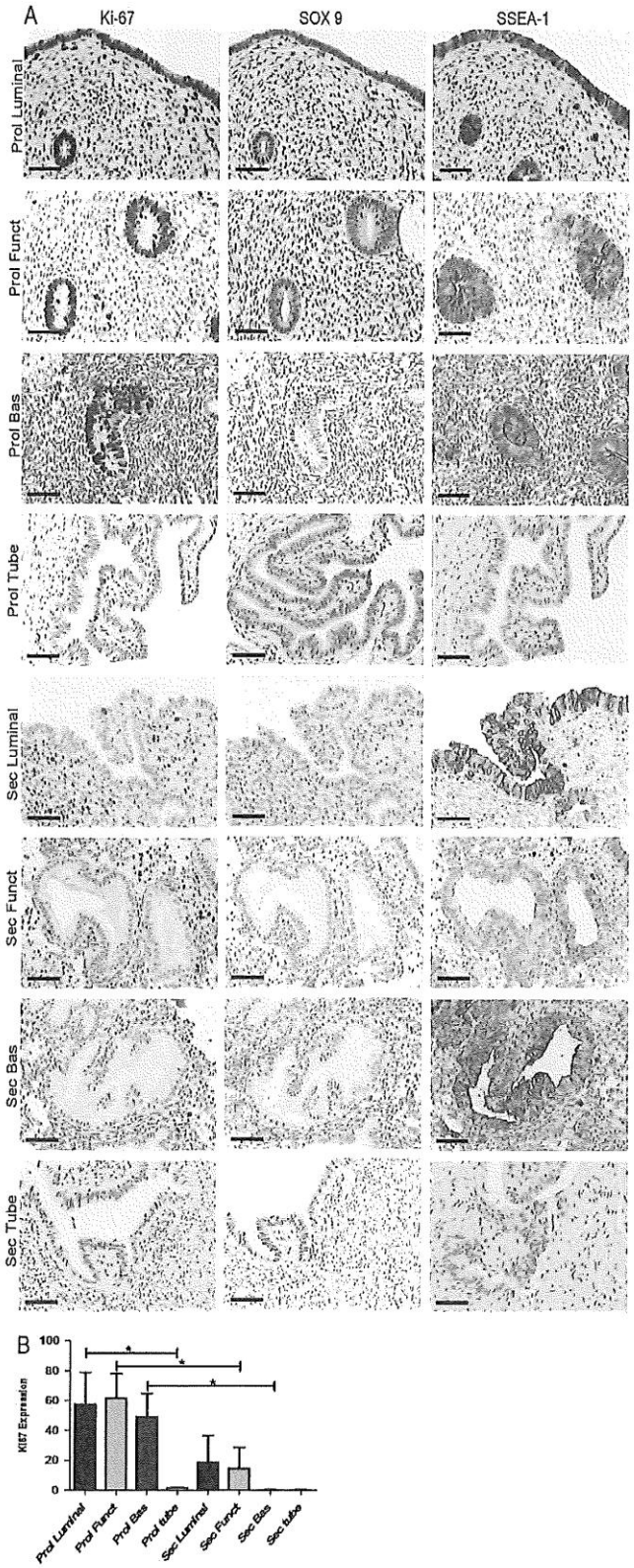

Figure 2 Ki67-Labelling Index (Ki67-LI) correlated with endometrial epithelial cell proliferation only in the epithelial compartment of the stratum functionalis. Epithelial *LGR5* expression scores also correlated with the expression of the previously known progenitor markers SOX9 and SSEA-1 in sequential tissue sections across the cycle. (**A**) Representative images of Ki67, SOX9 and SSEA-1 IHC in luminal, stratum functionalis, stratum basalis epithelial compartments of the eutopic endometrium and Fallopian tubes in the proliferative and secretory stages of the menstrual cycle (all images ×400, scale bar 10 μm). (**B**) Quantification

corresponding eutopic endometrium ($P < 0.01$, Fig. 1B). The cell type expressing *LGR5* was identified with ISH, demonstrating that *LGR5* expression was limited to the epithelial compartment in both endometrium and tube. Semi-quantitative scoring of *LGR5* expression revealed that the luminal epithelial cells expressed significantly higher levels of *LGR5* than all other epithelial compartments in the endometrium ($P < 0.05$, Fig. 1C and D) including the endometrial stratum basalis. The reduction in *LGR5* expression in the secretory phase was confirmed with ISH in the luminal ($P = 0.03$) and functionalis epithelium ($P = 0.04$) respectively (Fig. 1C and D).

LGR5 expression correlated with endometrial epithelial cell proliferation in the stratum functionalis epithelial compartment

The differences in the cellular proliferative activity in the three endometrial epithelial compartments across the menstrual cycle were demonstrated by the dynamic changes in the expression of the proliferative marker Ki67 (Fig. 2A and B) in sequential tissue sections. Epithelial Ki67-LI was highest in the proliferative phase with the maximum Ki67-LI observed in the cells of stratum functionalis glands (median 70%, range 10–100%) and the lowest Ki67-LI seen in the stratum basalis epithelium (median 30%, range 0–85%). Ki67-LI was higher in the stratum functionalis and in luminal epithelium compared with the stratum basalis glands in all phases of the cycle. In the secretory phase, Ki67-LI in all epithelial compartments decreased, with the luminal epithelial compartment demonstrating the highest Ki67-LI (median 1%, range 0–90%) and Ki67-LI was absent in the stratum basalis glands. Ki67-LI and *LGR5* expression levels only correlated significantly ($r = 0.74$, $P = 0.01$) in the stratum functionalis epithelium. The stratum basalis *LGR5* expression persisted in the secretory phase (Fig. 1D) while the corresponding Ki67-LI reactivity decreased significantly ($P = 0.03$) (Fig. 2B). The quiescent (absent Ki67-LI) atrophic postmenopausal endometrial epithelium also expressed *LGR5* (particularly the luminal epithelium) (Supplementary Fig. S2).

Epithelial proliferation (Ki67-LI) in the Fallopian tube was consistently very low throughout the cycle, contrasting with the dynamic tubal *LGR5* expression pattern ($r = 0.23$, $P = 0.55$).

In the human endometrium, luminal and basalis epithelia share distinct patterns of co-expression of *LGR5* and the previously known progenitor markers SSEA-1 and SOX9

Sequential tissue sections were employed to examine if the cellular location of *LGR5* mRNA (by ISH) was consistent with the expression of previously described endometrial basalis progenitor markers SSEA-1 and nuclear SOX9 (by IHC, Fig. 2A). In general, SOX9 and SSEA-1 expression followed the same cyclical pattern of expression as *LGR5*: levels decreased in all three endometrial epithelial compartments and also in the tubal epithelium in the secretory phase when compared with the samples from the proliferative phase (Fig. 2A). However, out of all three endometrial epithelial compartments, the strongest *LGR5* expression was seen in the luminal epithelium (Fig. 1C and D) whereas the strongest SSEA-1 and SOX9 staining was observed in the stratum basalis glands agreeing with previous reports (Valentijn *et al.*, 2013) (Fig. 2A). It is noteworthy that the luminal staining for both SSEA-1 and SOX9 was consistently high throughout the cycle, even when their expression decreased in the stratum functionalis epithelium in the secretory phase (Fig. 2A).

In the Fallopian tube, SOX9 staining scores and *LGR5* ISH scores were high throughout the menstrual cycle, similar to the stratum basalis glands of the endometrium with only an apparent reduction in the intensity during the secretory phase (Fig. 1D). In contrast, SSEA-1 scores were very low in the tubal epithelium in all phases of the cycle. The co-expression of SSEA-1 protein and *LGR5* mRNA by ISH was further confirmed with immunofluorescence staining (Supplementary Fig. S3).

Progestogens regulate *LGR5* expression *In vitro* and *in vivo*

The progestogenic regulation of *LGR5* expression was examined by treating endometrial explants *in vitro* with the synthetic progestogen MPA in short-term culture and MPA treatment decreased *LGR5* levels by 1.5-fold (Fig. 3A).

The *in vivo* effect of progestogens on the endometrial expression of *LGR5*, was tested in endometrial samples from women taking synthetic progestogen treatment (progesterone only pill, 'POP', or levonorgestrel-releasing intrauterine system, 'LNG-IUS') and a significant reduction of *LGR5* mRNA levels was observed with progestogen treatment compared with the normal eutopic endometrium of women not on hormonal treatments ($P < 0.01$, Fig. 3B). Even with these very low levels, the luminal epithelium continued to retain higher *LGR5* expression than the glands following progestogen treatment (Fig. 3C and D).

LGR5 expression did not correlate with epithelial cell proliferation in progesterone treated human endometrial samples

The luminal and glandular epithelial Ki67-LI was much higher in the POP samples when compared to the LNG-IUS treated endometrium (POP luminal median 50%, range 40–75%, glands median 70%, range 50–85%, LNG-IUS luminal median 1%, range 0–1%, glands median 2% range 0–2%, Fig. 4A and B). Therefore, the Ki67-LI levels in the POP treated samples did not correlate with the levels of *LGR5* expression, whereas in the atrophic glandular and luminal epithelial cells of the LNG-IUS samples there was low levels of both *LGR5* and Ki67-LI.

In silico analysis of published microarray data revealed potential *LGR5* regulating genes confirming progestagenic control

One hundred and thirty-three out of the 331 potential *LGR5* regulating transcription factors (TFs) were differentially regulated in the progesterone

of percentage Ki67-positive cells (Ki-67 LI) throughout the cycle. A minimum of 25 fields of cells were counted at ×400 magnification ($n = 7$ per group). The stratum functionalis in the proliferative phase has the highest Ki67-LI and is statistically higher than the stratum functionalis epithelium in the secretory phase ($P < 0.05$). Ki67-LI decreased dramatically in the secretory phase of the cycle in all epithelial compartments (Prol = Proliferative, Sec = Secretory Funct = stratum Functionalis, Bas = stratum Basalis).

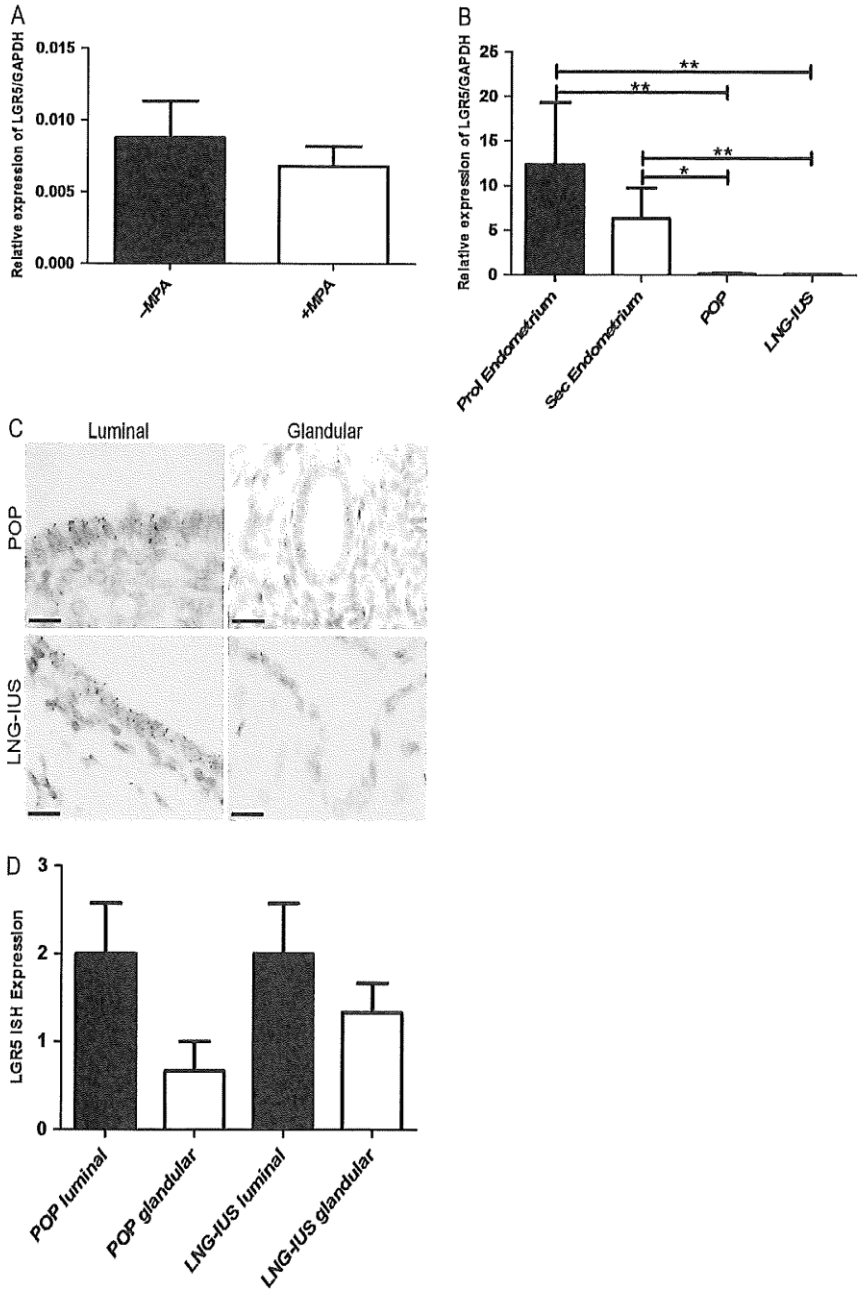

Figure 3 Progestagens regulate LGR5 expression *In vitro* and *In vivo*. (**A**) *LGR5* mRNA expression levels analysed by qRT-PCR. Explants treated with MPA ('+MPA') expressed lower *LGR5* levels relative to GAPDH when compared with the same of vehicle-treated explants ('–MPA') ($n = 6$ per group). (**B**) *LGR5* mRNA expression by qRT-PCR. Patients taking the oral progesterone only pill (POP) or having the levonorgestrel-releasing intrauterine system (LNG-IUS) have significantly less *LGR5* mRNA expression relative to GAPDH when compared with normal eutopic proliferative and secretory endometrium ($P < 0.01$ and $P < 0.04$, respectively). Untreated and progesterone treated (POP/LNG-IUS) ($n = 6$ per group). (**C**) Representative *LGR5* ISH images of POP treated and LNG-IUS treated luminal and glandular eutopic endometrium (All images ×1000, scale bar = 20 µm). (**D**) Graphical representation of semi-quantitative scoring of *LGR5* ISH. The luminal epithelium has more LGR5 when compared with the glandular epithelium in the POP and LNG-IUS treated samples ($n = 6$ per group).

dominant secretory endometrium when compared with the proliferative endometrium (Supplementary Tables SII and III), supporting a role for progesterone in the regulation of *LGR5* expression. The *LGR5* gene promoter has high-affinity binding sites for the progesterone receptor, suggesting a direct regulation. The sorted human endometrial epithelial side population cells (enriched for stem cells) showed differential expression of 48 TFs that potentially regulate *LGR5* compared with the unsorted differentiated epithelial cells (Supplementary Table SIV). The analysis of the upstream

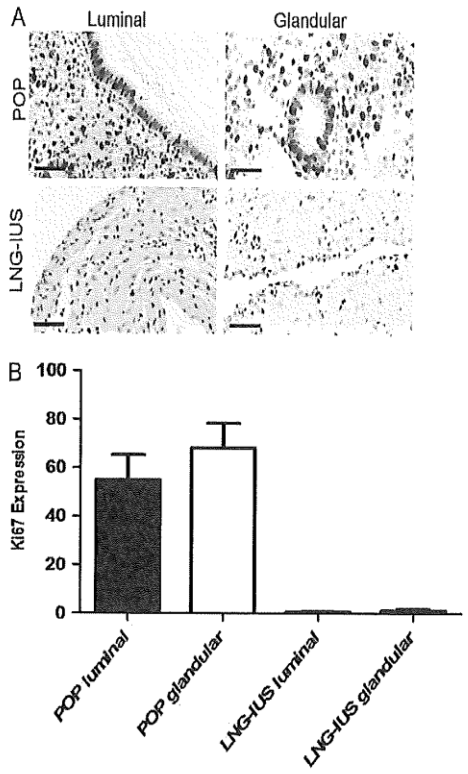

Figure 4 *LGR5* expression did not correlate with epithelial cell proliferation in progesterone treated human endometrial samples. (**A**) Ki67-LI IHC data, in the progesterone only pill (POP) group, the glandular and luminal epithelium showed high Ki67-LI but the levonorgestrel-releasing intrauterine system (LNG-IUS) group endometrial Ki67-LI was very low. (**B**) Representative Ki67 images of luminal and glandular eutopic endometrium from patients treated with either POP or LNG-IUS (all images ×400, scale bar = 10 μm, n = 6 per group).

regulating drugs and chemicals in IPA core analysis of the *LGR5* regulating genes that are differentially expressed in both side population epithelial cells and in the secretory phase endometrium identified progesterone, confirming our *in vitro* and *in vivo* wet-lab data (Supplementary Fig. S4) suggesting a role for progesterone in *LGR5* gene expression.

Discussion

This is the first comprehensive study employing the gold standard method; ISH, in order to examine the cellular location of LGR5 expression in full thickness normal human endometrium. High *LGR5* expressing cells were seen in the endometrial luminal epithelium and in the stratum basalis. Healthy human endometrium shows a dynamic spatiotemporal pattern of *LGR5* expression, suggesting hormonal regulation. Endogenous and exogenous progestogens appear to inhibit *LGR5* expression in the endometrium both *in vitro* and *in vivo*, and these data and *in silico* analysis of published endometrial microarray datasets were in agreement.

Previous evidence from other epithelial tissues proposes *LGR5* expression to be limited to stem cells and thus for *LGR5* to be an epithelial stem cell marker (Kumar *et al.*, 2014). We have shown that *LGR5* was not localised to a small number of cells in the adult endometrial basalis epithelium; the proposed location of the stem cell niche (Valentijn *et al.*, 2013; Gargett *et al.*, 2016). The *LGR5* expression we have seen in the human adult endometrium, unlike in the small intestine, mimics the *Lgr5* expression pattern seen in mouse uteri (Sun *et al.*, 2009). A uniform expression of *Lgr5* was seen in the ovariectomised uterine epithelium and it is suggested that most of these remaining epithelial cells have the potential to proliferate when necessary for uterine glandular growth (Sun *et al.*, 2009). A mouse endometrial epithelial organoid system, which allowed long-term expansion of epithelium, also showed *Lgr5* gene expression (Boretto *et al.*, 2017). In contrast, in humans, the whole of the endometrial functional layer is regularly shed with menstruation, a phenomenon not relevant to most mammals including rodents. The initial step of the embryo attachment and implantation occurs at the luminal epithelium, which exists at a relatively distant location in cellular terms from the stratum basalis (up to 16 mm in the mid-secretory phase). Due to external assaults such as mechanical friction or infection, cells are continually lost and replaced from the surface of any epithelial tissue including the skin and intestine (Barker, 2014); therefore a similar daily cellular loss is likely to happen at the endometrial luminal epithelium which is exposed to the uterine cavity and external environment. The daily maintenance of this luminal epithelium may require locally positioned cells with progenitor ability. Supporting this hypothesis, rapid *Lgr5*+ epithelial cell proliferation can be observed in many other organs upon tissue damage (Beumer and Clevers, 2016; Ng *et al.*, 2014).

We therefore hypothesise that it is possible for the human endometrium to have more than one epithelial stem/progenitor cell pool; one residing in the basalis (SSEA-1++SOX9++*LGR5*+) supporting the massive regeneration of the functionalis after menstrual shedding or parturition; while the other (*LGR5*++SSEA-1+SOX9+) supports the embryo-implantation process, and maintains the luminal epithelial cells that are likely to be lost on a daily basis (Fig. 5). This is in agreement with the scanning electron microscopy studies of human endometrium, the endometrial injury model of the rabbit (Ferenczy and Richart, 1974) and neo-natal endometrial glandular development in humans (Cooke *et al.*, 2013). The persistent expression of the progenitor cell markers SOX9 and SSEA-1 in the luminal epithelia, with concomitant high *LGR5* expression, corroborate further with the above hypothesis (Barker and Clevers, 2010; Valentijn *et al.*, 2013). Future work examining the functional properties of endometrial epithelial cell subpopulations that are isolated from the two anatomical regions within the human endometrium, which express either high or low LGR5, SOX9 and SSEA-1, is warranted.

The two antibody based studies examining LGR5 in the human endometrium (Gil-Sanchis *et al.*, 2013; Cervello *et al.*, 2017) are in contrast to our work, in that we do not detect *LGR5* expression in the stroma but only in the pancytokeratin expressing epithelial cells and the observed proportion of epithelial cells expressing *LGR5* exceeded 1%. It should be noted that mRNA and protein levels may not necessarily correlate, and in our hands, the endometrial LGR5 protein expression using two commercially available antibodies, demonstrated non-specific staining (Supplementary Fig. S5). Therefore, agreeing with the general consensus, we concluded that the reliability of antibodies against LGR5 remains in *considerable* doubt.

Suppression of glandular regeneration and progenitor activity is postulated to occur within the progesterone-dominant, non-proliferative,

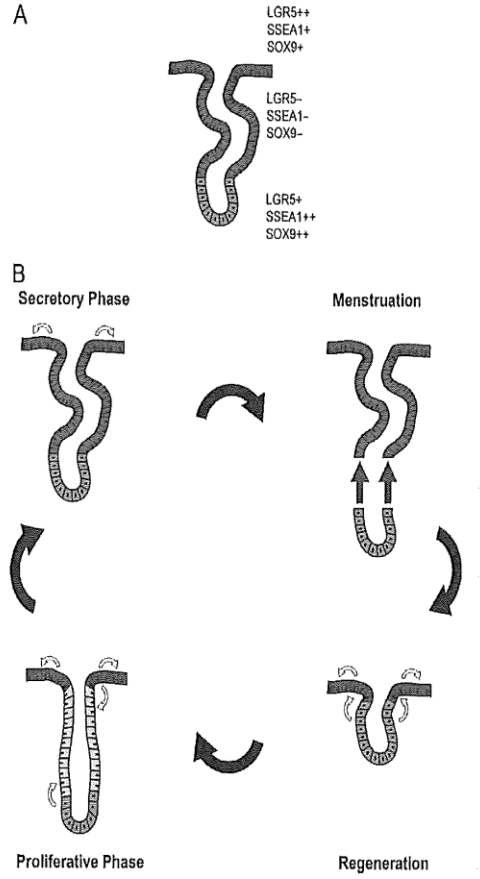

Figure 5 Putative model of endometrial epithelial regeneration. (**A**) The secretory phase endometrial epithelial configuration; luminal epithelia contains *LGR5*++SOX9+SSEA-1+ cells, functional glands contain *LGR5*-SOX9-SSEA-1- epithelial cells and basalis glandular cells with SOX9++SSEA-1++*LGR5*+ phenotype. (**B**) Luminal and functional layers are shed at menstruation, luminal epithelium regenerated from the stratum basalis epithelium (SOX9++SSEA-1++*LGR5*+) after menstrual shedding, subsequently, the luminal epithelium (*LGR5* ++SOX9+SSEA-1+) throughout the cycle regenerates itself and possibly also contributes to the regeneration of functional glands in the proliferative phase (*LGR5*+SOX9+SSEA-1+) whilst stratum basalis glands are responsible for the regeneration of all/most of the epithelia of the stratum functionalis in the proliferative phase.

secretory functional epithelium, where the lowest *LGR5* expression levels were observed. This is in agreement with a possible high *LGR5*-related stem/progenitor cell function and concurs with the *in silico* study demonstrating the differential expression of many potential regulators of *LGR5* gene in the stem cell enriched endometrial epithelial side population cells. Our interrogation of the published microarray datasets, also sought further information on the effect of progesterone on endometrial *LGR5* gene expression. We identified binding sites for progesterone, oestrogen and androgen receptors in the *LGR5* gene promoter and potential other *LGR5* gene regulators were also differentially expressed in the secretory phase endometrium. IPA core analysis re-confirmed the direct influence of progesterone on many of the identified differentially expressed *LGR5* regulators in the secretory endometrium. Considering the intricate relationship between steroid hormone receptors and their function, our experimental and *in silico* analysis data thus suggest that progesterone may directly and also indirectly regulate *LGR5* via downstream target genes. Endometrial epithelial differentiation, proliferative quiescence and inhibition of the canonical Wnt pathway in the stratum functionalis layer are known functions of progesterone (Wang *et al.*, 2009) and they were also identified as significant canonical pathways involving the differentially expressed (progesterone regulated), *LGR5* regulators in the secretory phase endometrium. This suggests a possible functional involvement of *LGR5* in the secretory endometrium that requires exploration in future studies.

In the absence of validated lineage markers for the various epithelial populations that are likely to exist within the endometrium, we cannot formally characterise the resident *LGR5*$^{+}$ cells as multipotent. Lineage tracing studies need to be completed in the human endometrial epithelium to identify the location of stem cells, this will further complement the *in vitro* functional studies to confirm if *LGR5* expressing epithelial cells indeed represent the epithelial stem cell population.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

N.T. and D.K.H. conceived the study, designed and performed the experiments, interpreted data, and wrote the first draft of the manuscript. N.A.W. and A.M.B. assisted with ISH experiments and revised the manuscript critically for important intellectual content. All authors revised and read the manuscript and approved the submitted final version.

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Conflict of interest

No conflicts of interest.

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3D Architecture of Endometrial Glands in Relation to Stem Cell Organisation. Nicola Tempest†,2 Ann-Marie Baker†,1 Marnix Jensen*,1 Nicholas Wright*,1 Dharani Hapangama*.2
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INTRODUCTION: Menstrual shedding and repair of the endometrial functionalis is unique to humans and higher-order primates. The current dogma is that endometrial epithelium regenerates from endometrial epithelial stem cells (EESC) residing in the base of the glands. This theory presumes endometrial glands to have a single tubular architecture. The precise 3D organisation of the human endometrial glandular epithelium is unknown. The existence of a human EESC is yet to be directly confirmed. Hypothesis: Human endometrium harbours EESC(s) with the ability to regenerate all endometrial epithelial cells; they reside in the base of the glands at the endometrial-myometrial junction that assume a blunt-ended tubular 3D architecture.

METHODS: The gold standard method, in vivo cell lineage tracing, with mitochondrial DNA mutations (mtDNAm) as clonal markers, was used to confirm the existence of EESCs. 100 consecutive paraffin sections stained with H&E / cytochrome *c* oxidase (CCO) from normal pre (menstrual/ proliferative/secretory) and postmenopausal endometrium were scanned and registered in FreeD16 for 3D reconstruction, manual drawing of gland boundaries in each 2D serial image were connected along the third dimension between adjacent slides.

RESULTS: A unique CCO-mtDNAm, in a partially mutated gland within a CCO deficient area confirmed a clonal population evidently with its origin in a stem cell (figure 1a). Basalis glands inter-connect (branch) and take a horizontal course along the myometrium (figure 1b); whilst non-branching single functionalis glands appear to germinate perpendicularly from these basal glands (figure 1c). mtDNAm occur in the basalis glands, allowing the vertical tracking of such mutations along the functionalis glands, confirming that at least one population of EESC resides in the basalis glands, responsible for endometrial glandular regeneration (figure 1d & 1e).

CONCLUSION: There is more than one EESC contributing to the endometrial epithelial regeneration. Our data demonstrates the complex 3D histo-architectural arrangement of human endometrial epithelium for the first time, highlighting that endometrial epithelium cannot be treated as a single entity. The suggested existence of multiple EESC with possible functional diversity warrants further interrogation which is essential for understanding endometrial pathology.

**Figure(s) will be available online.*

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Hormones and endometrial carcinogenesis

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Abstract: Endometrial cancer (EC) is the commonest gynaecological cancer in the Western World with an alarmingly increasing incidence related to longevity and obesity. Ovarian hormones regulate normal human endometrial cell proliferation, regeneration and function therefore are implicated in endometrial carcinogenesis directly or via influencing other hormones and metabolic pathways. Although the role of unopposed oestrogen in the pathogenesis of EC has received considerable attention, the emerging role of other hormones in this process, such as androgens and gonadotropin-releasing hormones (GnRH) is less well recognised. This review aims to consolidate the current knowledge of the involvement of the three main endogenous ovarian hormones (oestrogens, progesterone and androgens) as well as the other hormones in endometrial carcinogenesis, to identify important avenues for future research.

Keywords: androgens; endometrial cancer; gonadotropin-releasing hormones; oestrogen; polycystic ovarian syndrome; progesterone.

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Introduction

Endometrial cancer (EC) is the commonest gynaecological cancer in Europe and has an alarmingly increasing incidence related to longevity and obesity [1, 2]. The European estimates suggest, by 2025, an incidence increase of EC in the range of 50%–100% will occur, relative to the observed incidence in 2005 [3]. Many women with EC are postmenopausal (PM) and not suitable for standard surgical treatment because of other commonly coexisting medical problems, such as obesity, hypertension and diabetes. Latest national figures in the UK show that one in four women undergoing major gynaecological surgery for cancer experience serious surgical complications [4]. The 5-year survival rates for advanced EC are 23%, worse than for other common gynaecological cancers, and similar to that of ovarian cancer [5]. Current therapeutics fail to treat late stage disease, and preventing advanced disease and distant metastasis needs early diagnosis of ECs. Identification of those ECs likely to metastasise and treating them with adjuvant therapies is imperative to tackle the impending economic and social burden caused by the increasing incidence of EC predicted over the next decade [3].

Ovarian hormones regulate normal human endometrial cell proliferation, regeneration and function, therefore are implicated in endometrial carcinogenesis. Furthermore, recent evidence suggests that all types of ECs may share common etiological factors, including their response to/stimulation by oestrogen and other ovarian steroid hormones [6]. Although many reviews previously describe the role of oestrogen and progesterone in endometrial carcinogenesis, they have largely disregarded the involvement of androgens and other hormones. Recent reports however, describe a role for androgens and its receptor [7, 8], gonadotropin-releasing hormone (GnRH) and luteinizing hormones (LH) in EC [9, 10]. This review aims to explore the current knowledge on how endogenous and exogenous hormones, influence endometrial carcinogenesis.

Method

We carried out PubMed (Medline) and Ovid searches systematically for publications from November 2000 until November 2015. Keywords

used included: EC with hormones, risk factors for EC, obesity, adipose, polycystic ovarian syndrome (PCOS), lynch syndrome (LS), diabetes, Parkinson's disease (PD), pregnancy, menarche, menopause, combined oral contraceptive pills (COCPs), oestrogens, progestogens, selective oestrogen receptor modulators (SERMs), tamoxifen, selective progesterone receptor modulators (SPRMs), aromatase inhibitors, fulvestrant, hormone replacement therapy (HRT), GnRH analogues/antagonists, LH, danazol and androgens. All studies investigating hormonal influences in EC in women, animals and endometrial cell lines, either primary cells or tissue explants in culture were considered. Further manuscripts published before November 2000 were also reviewed for specific topic areas and included as appropriate.

Human endometrium as a hormone receptive organ

Anatomy/histology

Embryonic Mullerian ducts fuse in the midline to form the human uterus and the inner layer of its mesenchyme forms the endometrium. Initially, the superficial endometrial glands originate from the fetal undifferentiated, single columnar epithelial cells in utero. The histological architecture of the endometrium at birth is analogous to the PM endometrium yet the glands remain to be shallow and superficial [11]. Humans (and other upper order primates) menstruate, and have two distinct layers of the endometrium, making them unique, amongst other mammals. The transient superficial stratum functionalis exists only during the reproductive life of the woman whilst the structurally permanent deeper stratum basalis adjacent to the myometrium remains throughout life [12]. Endometrium consists of a variety of cell types including epithelial, stromal, endothelial and leucocyte cell populations. As most ECs are carcinomas, in the context of carcinogenesis, the critical endometrial cell type is the epithelial cells. Endometrial epithelium is also organised into distinct groups of cells (i) luminal epithelium, that lines the superficial surface of the uterine cavity, (ii) the hormone responsive, fully differentiated functionalis glands and (iii) the deeper SSEA-1 expressing basalis glandular epithelium [13]. Presumably, a new functionalis layer is generated each month from the remaining basalis after menstrual shedding, hence the basalis is proposed to harbour stem/progenitor cells (SPCs) [12]. ECs commonly occur in the PM endometrium, which is essentially the retained luminal and basalis epithelium. SPCs are thought to play a role in carcinogenesis and these epithelial layers are also their postulated location [14]. The particular endometrial epithelial cell group(s) that gives rise to ECs is not yet known. However, we can conclude that either luminal or basalis glandular compartments are likely to be the common epithelial origin of ECs.

Overview of normal endometrial endocrinology

Endometrium is the primary recipient organ for ovarian steroid hormonal signal and is intricately responsive to these hormones. The three main classical ovarian steroid hormones, oestrogens, progesterone and androgens exert their effects in the endometrial cells mainly via their cognate receptors [12, 15, 16]. Endometrium expresses both subtypes of classical nuclear oestrogen receptors (ERα and ERβ). Progesterone, acts via the progesterone receptor subtypes PRA and PRB. Androgens operate via androgen receptor (AR) and all these receptors belong to the Class I nuclear hormone receptor superfamily of ligand-inducible transcription factors that share the common, evolutionarily conserved structural and functionally distinct domains of other superfamily members [12]. These classical hormone receptors are activated upon ligand binding and may exert effects involving the classical hormone-signalling pathway [12, 15]. This involves steroid receptor dimerising, translocating to the nucleus, binding to the respective hormone responsive element located in the relevant gene promoters to initiate recruitment of co-activators, co-repressors and chromatin remodelling factors to either activate or repress transcription of target genes. Post-translational modifications of the steroid hormone receptors will also affect gene promoter targeting and subsequent target gene transcription.

The general consensus is that oestrogen induces the expression of all endometrial steroid receptor types via the action of ERα whilst progesterone may downregulate them via PR (except for ERβ) [12, 17] (Figure 1). Androgens acting via AR induce their own receptor expression [18, 19] but AR-mediated effect on the other hormone receptors in the endometrium is not yet known. The expression profile of these different steroid receptors and the levels of respective hormones at the cellular level are therefore important to ascertain the full impact of hormones on the endometrium (Figure 1).

The intra-cellular levels of steroid hormone metabolising or activating enzymes may also play a pivotal role in regulating the final effect of these hormones [20]. To add another layer of complexity, some hormone receptors make heterodimers upon hormone/ligand binding. For example, ERα dimerise with ERβ and the effect of oestrogen displayed by cells expressing equal amount of both receptors

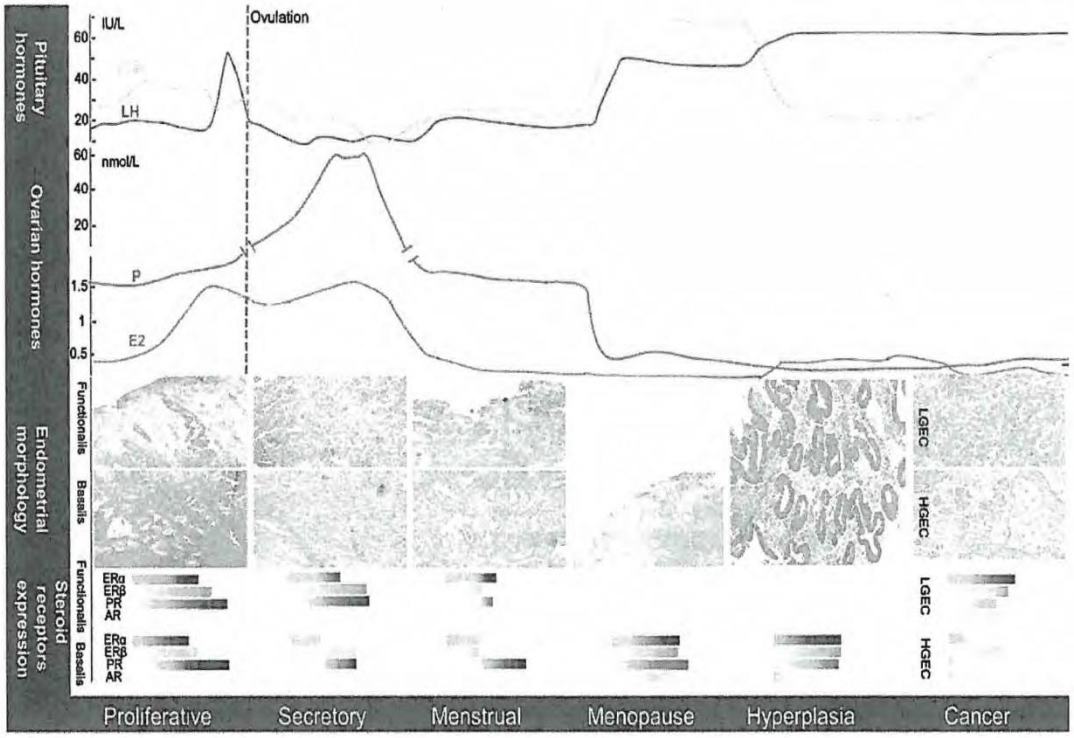

Figure 1: Correlation of typical hormonal changes of pituitary/ovarian axis with the consequential endometrial morphology and steroid hormone expression in women.
Pituitary and ovarian hormones show typical cyclical variations during the premenopausal period and these change at menopause. The correspondent endometrial morphology and epithelial steroid receptors expression in normal [pre and postmenopausal (PM)], premalignant [endometrial hyperplasia (EH) with atypia] and malignant (low-grade endometrial cancer, LGEC and high-grade endometrial cancer, HGEC) endometrium is shown in the panels.

will be different to another expressing more of a particular subtype of these receptors [12]. Furthermore, most class I hormone receptors can bind to the other ovarian steroid hormones with a lower affinity due to the significant homology between their ligand binding domains [21]. Therefore, when some steroid hormones are abundant, they may also exert some effects via a different steroid receptor, as well as acting through their specific cognate receptor.

Hormonal milieu of premenopausal endometrium

The prepubertal ovary is inactive, hence, the thin endometrium grows very slowly from birth until puberty due to the lack of ovarian hormonal (mainly oestrogenic) signal [12]. In response to the increasing levels in ovarian oestrogen and adrenal androgens observed at puberty, endometrium acquires the typical adult histo-anatomical configuration, and with the oestrogenic stimuli in the proliferative phase grows a functionalis layer that differentiates with the subsequent exposure to progesterone produced by the corpus luteum [22]. At the end of an infertile cycle, with luteolysis, the drop in progesterone (and oestrogen) initiates an inflammatory cascade, vasoactive and hypoxic events result in shedding of the functionalis as menstruation, followed by epithelialisation and regrowth of the functionalis to begin the next endometrial cycle [23] (Figure 1). Ovarian production of androgens is also reported to follow a cyclical pattern, yet the exact function of androgens in the normal endometrial function and regeneration is not fully described.

Hormonal milieu of PM endometrium

Similar to the prepubertal endometrium, the main histological characteristic of the PM endometrium is the complete loss of stratum functionalis. It is composed of inactive glands lying in compact stroma that morphologically resembles the stratum basalis of premenopausal endometrium [13, 24]. The PM hormonal milieu is characterised by the absence of progesterone and oestradiol production by the ovaries, persisting levels of androgens from the adrenals and the presence of low levels of circulating

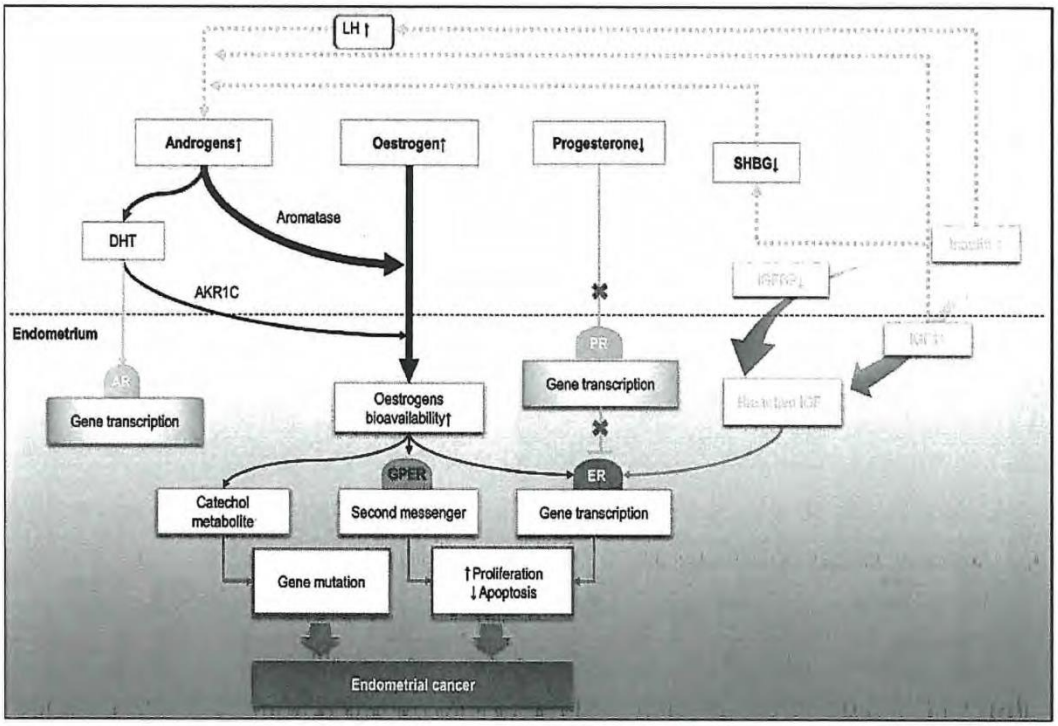

Figure 2: Different hormonal and metabolic pathways associated with disturbed steroid hormones homeostasis in favour of oestrogen pro-oncogenic pathway.
The figure illustrates how the aberrations associated with three major ovarian hormones, oestrogen, progesterone and androgens are involved in endometrial carcinogenesis.
AKR1C, aldoketoreductase1C; DHT, dihydrotestosterone; GPER, G protein coupled oestrogen receptor; IGF-1, insulin-like growth factor 1; IGFBP, insulin-like growth factor binding protein; LH, luteinising hormone; SHBG, sex hormone binding protein.

oestrone produced by the extra-ovarian aromatisation of adrenal androgens [20] (Figure 1). This low level of oestrogenic signal supports the maintenance of hormone receptor expression observed in the PM endometrium hence the hormone responsiveness [25].

Hormonal aberrations associated with EC

Oestrogen and insulin in excess and lack of progesterone have been proposed as the main hormonal aberrations to affect endometrial proliferation and cell survival that can result in increasing the risk of epithelial cell transformation/carcinogenesis. This section considers these hormonal aberrations in detail in the context of EC (Figure 2).

Unopposed oestrogens

As previously mentioned, oestrogen mediates endometrial cell growth, proliferation, apotosis inhibiton and angiogenesis in vitro and in vivo reviewed in [12]. ERβ may have opposing action on the classical ERα activity and is thought to play an antiproliferative role in endometrial proliferation [12]. Although not completely decoded, several pathways have been identified to exert oestrogenic action in endometrium, these include:

(i) Genetic pathway: An activated ER induces gene transcription either directly by binding to oestrogen response element (ERE), hence via "classical oestrogen response ERE dependent" route or indirectly "non-classical, ERE independent" path via interacting with transcriptional factors such as SP-1 [26], insulin-like growth factor (IGF-1) receptor [27], epidermal growth factor (EGF) receptor [28], PA-1 [29], NF-κB [30]. ER gene transcription can also be induced through downstream factors such as EGF or IGF-I via the "ligand independent pathways" [31, 32].
(ii) Non-genetic pathways: Oestrogen activation of ERK1/2 signalling pathway [33] or MEK/ERK, MAPK pathway [34] has also been demonstrated via calcium influx and membrane located non-classical ER (GPR30), respectively, in the absence of nuclear ER expression.

(iii) Epigenetic: Hypermethylation of ESR1 and PRB promoters has been reported in 90% of the EC [35–37]. Furthermore, hypomethylation with subsequent activation of oestrogen downstream PAX2 has been described in EC and associated with increased tumour growth in vivo [38].
(iv) Mutagenetic: Several lines of evidence have shown that 4-hydroxylated oestrogen (catechol-oestrogens), catalysed by cytochrome P450 1B1, is able to induce DNA damage [39]. Accumulation of catechol oestrogens is observed with prolonged unopposed oestrogen exposure. Interestingly, catechol oestrogens have been associated with DNA damage at a specific DNA region (codon 130/131) on PTEN gene which is frequently mutated in type 1 ECs [40].

It is clear that there is an overlap in the pathways that derive oestrogen pro-oncogenic environment and we can conclude that unopposed oestrogenic stimuli, even with low levels of oestrogen may adversely affect the endometrium.

Progesterone insufficiency

Progesterone counteracts the above-mentioned trophic drive of oestrogen, therefore anovulation, or lack of synthesis of endogenous progesterone by the ovary that can occur without a perturbation of oestradiol production, is associated with an excessive and prolonged proliferation of the endometrial cells and thickening/hyperplasia of the glandular epithelium. For example, telomerase enzyme is pertinent for endometrial epithelial proliferation, is highly active in endometrial hyperplasia [EH] and in cancer and is induced by oestrogen and inhibited by progesterone [41].

The antiproliferative action of progesterone in the endometrial epithelial cells is exerted directly and indirectly via the stroma. Apart from the progesterone receptor, androgen and glucocorticoid receptors are also postulated to be involved in progesterone action in the endometrium [42]. The evidence for the stromal involvement in the antiproliferative action of progesterone comes from a tissue recombination study utilising PR knockout (PRKO) mice [43], and by selective inactivation of endometrial epithelial PR [44].

Thus, three possible mechanisms were proposed to explain progesterone-oestrogen antagonism (Figure 2) (i) via inhibiting ERα expression, (ii) via preventing stromal production of growth factors and subsequent transcription factor activation [45, 46] and (iii) via inducing apoptosis of endometrial epithelial cells through PR action in stromal cells [47].

Therefore, the deficiency of endogenous progesterone levels or diminished length of lifetime progesterone exposure (anovulation and lack of full-term pregnancy), are associated with the development of EH and increased risk of EC [48].

Hyperinsulinism

Hyperinsulinism, associated with either diabetes mellitus or PCOS, plays an important role in carcinogenesis as it potentiates mitotic activity in the glands and stroma by increasing the activity of IGF-1 [49, 50]. Insulin excess stimulates theca cell androgen activity, elevates serum free testosterone levels through decreased hepatic sex hormone-binding globulin (SHBG) production, amplifies LH and IGF-I-stimulated androgen production, and enhances serum IGF-I bioactivity through suppressed IGF-binding protein production [51, 52]. Insulin binding sites are also expressed in the endometrial stroma of women with EC [53] (Figure 2). Therefore, evidently, an excess in insulin signalling can result in endometrial changes with a pro-proliferative, pro-survival phenotype and inflammatory changes akin to unopposed oestrogen as mentioned above.

Hyperandrogenism

Although hyperandrogenism has been debated as a cause for the increased EC risk in women with PCOS, the in vitro and in vivo evidence to support carcinogenesis effect of androgens in the endometrium is weak [54].

Several studies have shown an increase in circulating androgen levels in EC patients nonetheless these studies have not ratified the EC risk after adjusting for the oestrogen levels. There are two androgen regulatory pathways active in the endometrium (Figure 2) (i) androgenic pathway via androgen receptor and (ii) oestrogenic pathway via ERs when androgens (androstendione and testosterone) are aromatised locally to oestrogenic compounds. Unlike in the normal endometrium, dominance of the second pathway in EC can be explained by the abundance of aromatase [55, 56] and aldo-keto reductase (AKR1C) [57] enzymes in the neoplastic endometrial cells, that increase the local availability of oestrogenic ligand. This is further exagerated by the relatively higher affinity of oestrogens to their cognate receptors, compared with that of androgens to AR [58]. Interestingly, the expression of aromatase observed to be higher in type II EC compared to type I, which will allow this subtype of EC to increase

local oestrogen biosynthesis [59]. Emerging evidence also show AR to be a positive prognostic indicator and that its loss is associated with shorter disease-free survival [8, 25], therefore, androgenic pathway as a therapeutic target for EC requires further investigation.

Other hormones

GnRH

Altered or high levels of GnRH is known to have antiproliferative effect on the endometrium indirectly via its suppression of gonadotropin secretion and inhibition of ovarian oestradiol synthesis as well as working directly on the endometrium via GnRH receptor (GnRH-R). In vitro, GnRH has been shown to stimulate apoptosis in normal endometrial cells [60]. GnRH-R are expressed in breast and gynaecological tumours, wherein their activation by agonists results in antiproliferative, antimetastatic and antiangiogenic effect [9].

Luteinizing hormone/human chorionic gonadotropin

LH/hCG receptors (LH-R) are expressed in 80% of ECs [10, 61] in a grade-specific manner and may regulate the invasiveness of EC cells [62]. The overexpression of the LH-R increases the ability of EC cells to undergo local invasion and metastatic spread in animal models. Likewise, LH withdrawal strongly inhibits local and distant metastatic spread of tumors [63]. LH upregulates its own receptor, therefore it is an important target in relation to the PM period where the levels of LH remains elevated (Figure 1).

Prolactin (PRL) and thyroid stimulating hormone (TSH)

Pituitary hormones PRL and TSH may influence ovarian production of sex hormones. These hormones may have a potential to be used as serum biomarkers to detect EC patients from healthy controls with 98.3% sensitivity and 98% specificity [64]. When combined in a five marker panel (including PRL, growth hormone, eotaxin, E-selectin and TSH) it was possible to discriminate EC from ovarian and breast cancers [65]. Hyperprolactinemia is induced by the use of antipsychotics in premenopausal women, and antipsychotics have been proposed as an independent variable for risk of EC [66]. Further studies are needed to confirm these preliminary reports.

Melatonin

Melatonin (N-acetyl-5-methoxytryptamine), is a hormone produced primarily by the pineal gland, appears to protect against cancer development in general. Melatonin production is under regulation of the hypothalamus, with highest levels of melatonin secreted during the night and when sleeping. It is known to exert antioxidant, antimitotic, antiangiogenic activity in tissues, as well as being an immune modulator and a regulator of fat metabolism [67]. Melatonin interacts with membrane and nuclear receptors, and may be linked to the regulation of tumour growth [68, 69]. It is of particular relevance to EC as it may also block ERα and affect the activity of aromatase. Women with EC were found to have lower melatonin levels and a significantly increased relative risk of developing EC was reported in overweight night shift workers compared to lean women [70]. Melatonin administration in addition to HRT was associated with reduced body mass, intraperitoneal fat, reduced endometrial proliferation and prevented the appearance of histological atypia of the endometrium in an ovariectomised rat model, indicating melatonin may have a prophylactic role in preventing EC in PM women [71].

Endometrial cancer

Classification

Histological classification

The dualist model of EC proposed by several researchers has been widely accepted over the last three decades [72, 73]. Based on endocrine, clinical and histopathological characteristics, it categorises EC to type I oestrogen-dependent adenocarcinoma, associated with favourable

Table 1: Common molecular alterations differentially associated with dualistic EC classification.

Type I		Type II	
PTEN mutation	52%–78%	TP53 mutation	60%–91%
PIK3CA mutation	36%–52%	PIK3CA mutation	24%–42%
PIK3R1 mutation	21%–43%	PPP2R1A mutation	15%–43%
KRAS mutation	15%–43%	HER2 amplification	27%–44%
ARID1A mutation	25%–48%		
CTNNB1 mutation	23%–24%		
Microsatellite instability	28%–40%		

outcome and endometrioid morphology and type II non-oestrogen-dependent EC, with worst outcome and serous papillary or clear cell morphology. Although this model was broadly supported by the reported specific molecular aberrations in each type (Table 1) [74, 75], it failed to define high grade type I endometrioid EC which showed a molecular pattern and outcome that blends between type I and type II EC [76]. Moreover, the persistent expression of steroid receptors, ER and to lesser extent PR in these so-called non-oestrogen-dependent EC [76–78] suggests hormonal regulation of Type II EC.

Molecular classification

The heterogeneity and overlap of the reported morphological and molecular abnormalities between different groups of ECs have restricted the traditional postsurgical risk group stratification (low, intermediate and high-risk group) and limited its prognostic and predictive value [79]. The recent emergence of a large cohort of endometrioid, serous and mixed ECs and subsequent reports of integrated proteomic and transcriptomic profile of them has added a new perspective to EC taxonomy [80]. The Cancer Genome Atlas (TCGA) study identified four genomic subgroups: POLE ultramutated (favourable outcome), microsatellite instability, copy number low (both medium risk/outcome) and copy number high (the worst outcome). This classification is in harmony with the dualistic model whereby POLE ultramutated, microsatellite instability and copy number low groups comprise 97% of type I EC while the copy number high group encompasses 94% serous EC. Nonetheless, 24% of grade 3 endometrioid were also resolved to the worst outcome, high copy number group confirming the previously predicted overlap between the subtypes [80]. This warrants integration of clinic-pathological, molecular and genomic parameters and a more holistic approach in the future to improve postsurgical stratification and tailoring personalised adjuvant therapy for women with EC to improve outcome [81].

Hormonal influence of the established risk factors of EC

The role of steroid hormones, principally oestrogen, in the aetiology of EC has been confirmed by experimental, clinical and epidemiological studies. Risk factors such as early menarche, late menopause, nulliparity (particularly when associated with ovulatory dysfunction) are directly linked to prolonged unopposed oestrogen exposure [82] yet indirect involvement of steroid hormone and imbalance that occur with other known risk factors has also been reported. This section highlights the possible contributions of steroid hormones to these widely accepted EC risk factors (Figure 3).

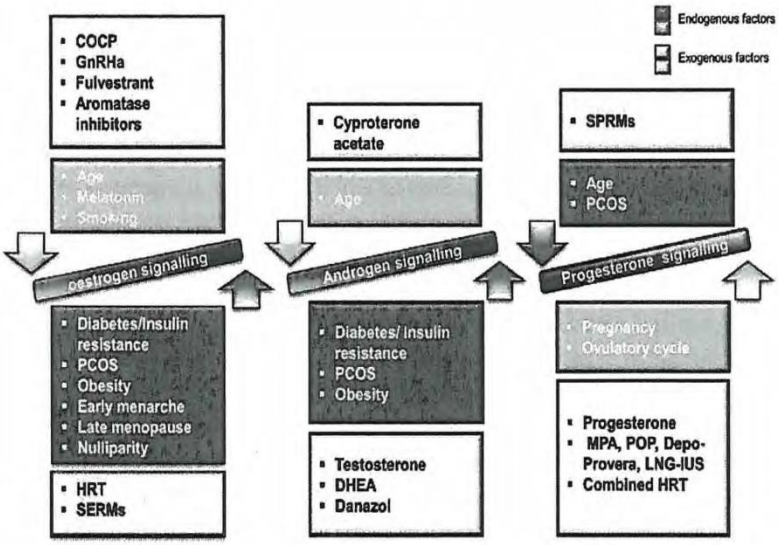

Figure 3: Endogenous and exogenous factors that affect steroid hormone signalling and contribute to endometrial carcinogenesis in the human endometrium.
COCP, combined oral contraceptive pills; DHEA, dehydroepiandrosterone; GnRHa, gonadotrophic-releasing hormone analogues; HRT, hormone replacement therapy; LNG-IUS, levonorgestrel-releasing intrauterine system; MPA, medroxyprogesterone acetate; PCOS, polycystic ovarian syndrome; POP, progestogen only pills; SREMs, selective oestrogen receptor modulators; SPRMS, selective progesterone receptor modulators.

Polycystic ovarian syndrome

The first report proposing a possible association between PCOS and EC appeared in 1949 [49]. Women with PCOS appear to have a three to four-fold increased risk for EC [83, 84], which translates to a lifetime risk of 9%, given the background lifetime risk of 3% in the general population [85].

PCOS is a heterogeneous hormone-imbalance disorder [86] and the commonest endocrine disease of women of reproductive age, affecting 5%–10% [87]. The pathogenesis of PCOS is multifactorial and is far from being fully understood [53]. It is a leading cause of anovulatory infertility and therefore is associated with the primary hormonal aberrations of unopposed oestrogen action, insulin resistance, hyperandrogenism and clinical features of diabetes and obesity [88, 89]. For the purpose of this review, we focused on the PCOS associated hormonal changes that have shown a causative relationship with EC, except the hormonal disturbances associated with obesity (which are independent risk factors of EC and discussed in detail in a later section).

In women with PCOS, the endometrium tends to remain in an oestrogen-mediated proliferative state due to chronic anovulation [53]. ERα and steroid receptor co-activators are overexpressed, signifying a reduced effectiveness of progesterone [90] and possibly contributing to a high sensitivity to oestrogen action [91].

The molecular mechanisms underlying endometrial progesterone resistance or sensitivity in these patients are not completely understood [86]. Impaired follicle maturation and consequent anovulation cause a chronic progesterone deficient state that affects the endometrial milieu. Alterations in the expression or function of PR co-activators, chaperones and co-chaperones that are bound to PR before activation and changes in gene expression are implicated in progesterone resistance [86, 90]. However, the classic progesterone resistance is proposed to describe the pathogenesis of endometriosis [92], and endometriosis is not associated with an increased risk of EC. Therefore, the contribution of progesterone resistance phenomenon in the pathogenesis of PCOS associated EC is debatable.

Hyperinsulinemia, a common finding in patients with PCOS regardless of body mass index (BMI) [51, 86] and anti-diabetic medications such as metformin have been used to normalise the hyperinsulinism in PCOS.

Hypersecretion of LH is also a frequent feature of PCOS and a modulator of endometrial growth, as evidenced by the ability of LH to promote growth of human EC cells in vitro. Endometrial LH receptors are overexpressed in anovulatory PCOS women with EH or EC [51].

Hyperandrogenism, as a cardinal feature of PCOS, is a common finding in EC [51]. Pituitary LH hypersecretion results in excessive theca cell stimulation and PCOS patients tend to have genetically impaired theca cell functional defect. Consequent hyperandrogenism [93] results in increased bioavailability of unopposed oestrogens due to the increased peripheral conversion [53, 94]. Peripheral aromatisation of androgens occurs preferentially in the adipose tissue and high BMI is a common feature of PCOS. This makes PCOS a self-propagating, oestrogen driven, hypermitotic condition of the endometrium. Therefore, it is obvious how all the above-mentioned hormonal changes associated with PCOS may increase the risk of EC. Normalising the hormonal aberrations with life style changes, limiting calorie intake, increasing exercise, weight loss and progesterone therapy to prevent EH may reduce the risk of EC.

Endometrial hyperplasia (EH)

EH is a pathologically heterogeneous diagnosis that ranges from histologically subtle and spontaneously reversible proliferative lesions to premalignant changes [95, 96]. It virtually always results from prolonged oestrogen stimulation, unopposed by progesterone. EH with cytological atypia is recognised as the precursor for the endometrioid type of EC, with significantly increased EC risk (RR=14, 95% CI, 5–38) [97]. Altered circulatory hormone levels are reported in EH patients, particularly in those with metabolic syndrome with elevated oestrogen, testosterone, insulin, leptin, LH and LH/FSH ratio [98, 99]. Additionally, increased oestrogen bioavailability as a consequence of increased local aromatase expression and activity, in hyperplastic endometrium has also been postulated [100]. Likewise, a significant change in AKR1C3, an enzyme catalyses progesterone, oestradiol and androstenedione, has been shown in EH compared with normal control and this is suggested to increase oestrogen signalling. However, there are conflicting reports of AKR1C3 expression profiles needing future clarification [57, 101]. Insulin resistance and carbohydrate metabolism disturbances are also common findings in EH [102], which demonstrated an increase in the mitogenic effect of oestradiol through EGF and activated IGF-1 [98] (Figure 2). Recently, reduced serum melatonin level has been described to predict EH transformation to the premalignant atypical phenotype [103].

Obesity

Excess weight has been shown to be causative for at least 40% of ECs in the UK [104, 105] and rather than BMI as a risk factor, the adult weight gain has been proposed as a better measure [106, 107]. The distribution of the excess adipose tissue within the body, specifically in visceral fat, is an independent EC risk factor [108].

There are three main theories linking obesity to EC (i) oestrogen, (ii) insulin and other growth factor production and (iii) excess production of inflammatory cytokines, adipokines in the visceral adipose tissue [109]. The most prominent out of these is the effects of unopposed oestrogen, due to aromatisation of adrenal androgens [107, 110]. Aromatase promoter in the adipose tissue is also under the influence of glucocorticoids and oestrogen-induced class 1 cytokines and TNFα [111].

Adipocytes produce adipokines such as adiponection and leptin. The serum leptin-adiponectin ratio is independently associated with increased EC development risk [112]. Adipokines may influence EC through mechanisms dependent/independent of oestrogen [113–115]. Adiponectin levels have shown to be inversely correlated with EC [116, 117] which might be due to polymorphisms in the adiponectin coding gene ADIPOQ [118]. Leptin is regulated by various hormones including insulin, glucocorticoids, TNFα and reproductive hormones [119]. Intentional weight loss has shown a decrease in several adipokines including leptin [120] and therefore it is expected to reduce EC risk.

Diabetes mellitus

There is a plethora of recent evidence suggesting that diabetes constitutes an independent risk factor for EC [121]. In addition to oestrogen-progesterone imbalance, which associates with obesity, the observed risk in type II diabetes can also be explained by the compensatory increase in the insulin production. This, consequently results in an increase in the circulating IGF-1 level [122] which acts as a mitogenic stimuli via non-classical oestrogen pathway, resulting in increasing the risk of EC [123].

Lynch syndrome (LS)

Approximately, 2%–5% of ECs may be due to inherited susceptibility, of which LS is the most common with an increased lifetime risk of EC 27%–70%. MLH1 mutations are frequent in EC but compared to colorectal cancers there is a five-fold increase in the prevalence of MSH6 mutations in EC [124–126]. Pathogenesis of LS is considered to be due to mutations of genes in the DNA mismatch repair (MMR) pathway [127]. An interesting theory proposes that MMR deficiency to be the most important abnormality in early stage EC [128]. High premenopausal level of oestradiol has shown to increase the MMR activity in vitro, which explains the lower incidence of EC in premenopausal women, whereas cells dividing in a low-oestrogen environment (such as in PM obese women) are more likely to accumulate genetic errors due to low DNA repair activity and they may be at high-risk for carcinogenesis [128]. Patients with defective MMR pathways are prone to microsatellite instability (MSI) and almost all LS-associated cancers have MSI due to MMR, but in some cases, it can also result from methylation of MLH1 promoter [129]. A later age of menarche, increased parity and the use of hormonal contraceptives may be protective against EC in LS women with MMR mutation [130]. As high levels of oestrogen may increase MMR proteins and thereby may play a protective role, combined pill in particular may be a useful chemo-preventive agent in these women at high-risk of developing EC.

Smoking

The inverse relationship between cigarette smoking and EC risk is well established [131]. According to a recent study, reduced EC risk was evident among former (RR=0.89, 95% CI 0.80, 1.00) and current (RR=0.65, 95% CI 0.55, 0.78) smokers compared with non-smokers [132]. This protective effect is independent of other EC risk factors and is fully explained via hormonal modulation affecting the function of hormone-producing organs, including adrenals and ovaries. Anti-oestrogenic mechanisms have been suggested through (i) increasing hepatic metabolism of oestrogen into minimally active 2-hydroxy-oestrogens [133], (ii) reducing ovarian oestrogen production which associates with a shorter follicular phase and early menopause [134, 135] and (iii) reducing aromatisation of androgens by the adipose tissue considering the positive association between smoking and lower BMI [132]. Adrenal androgen production in smokers is higher than in non-smokers [136] and smoking is associated with higher circulating androstenedione and testosterone levels [137, 138]. PM smokers with high BMI maintained a lower risk for EC [139]. Serum oestrogen level for these women were not measured in that particular study, but it is tempting to speculate that aromatisation of adrenal androgen in the adipose tissue of women who smoke may be impaired, which needs confirmation in future studies.

Parkinson's disease (PD)

Oestrogens are implicated in neurodegenerative diseases and PM HRT has been associated with lower risk of PD [140, 141]. Contrasting with EC, women with short menstrual span (late menarche, early menopause) seem to have higher risk of developing PD [142]. Furthermore, menstruation and pregnancy was associated with worsening of the symptoms and rapid progression of the disease. Consistent with this observation, reports from murine models have also shown that oestrogen and progesterone (but not testosterone) rescue substantia nigra from induced toxicity [143, 144]. Although this may suggest a protective effect of PD in EC, conversely, a recent report suggests that patients with PD have an increased risk in developing EC. This was accompanied by an overall reduction in risk of developing other cancers in general [145]. This association was not maintained after adjusting for multiple testing, therefore further studies will be required to confirm the causative relationship.

Role of common hormonal pharmacological agents in endometrial carcinogenesis

Pharmacological agents that operate via altering the hormonal activity in the body are considered here for their utility as preventative and treatments strategies or due to the role, they play in altering the risk of developing EC (Figure 3).

In support of that, the recent integrated genomic study [80] has identified a subset of hormonally responsive ECs that demonstrate alterations in hormonal pathways. Interestingly, the highest expression of ESR1 and PGR was found in copy number low and microsatellite instability groups rather than the favourable outcome POLE ultramutated group [80]. This further advocates personalised endocrine therapy in EC management. In this section, we discuss the therapeutic, preventative role of hormones as well as many of the commonly used hormonal agents in EC.

Oestrogen and progesterone based therapeutic modalities

As contraceptives

Since hormonal contraceptives are used by premenopausal women, yet EC is mainly a PM disease, any influence of hormonal contraceptives on endometrial carcinogenesis in general may be regarded as a delayed consequence.

Combined Oral Contraceptive pill (COCP): The protective effect in regards to EC has been well-documented [146]. The risk reduction appears to be proportionally related to the duration of use, and every 5 years of use is associated with a relative risk of 0.76 [147]. This favourable outcome may persist up to 30 years after cessation of COCPs, though it may be amplified as time after discontinuation elapses [148]. The similar protective effect observed in high and low dose oestrogen-containing pills [147] may suggest that the protective effect seen with COCP may be due to the increase in lifetime exposure to progesterone. In support of this finding, a higher progestin-potency COCP seem to be required for women with larger BMIs [149].

Progesterone only preparations: The use of progestogen only contraceptive agents such as depot medroxyprogesterone acetate (MPA) have been associated with a reduction in the risk of developing EC [150]. Likewise, the use of levonorgestrel-releasing intrauterine system (LNG-IUS) was found to be significantly protective with prolonged use up to 10 years [151].

High-dose systemic Depo-Provera preparation will induce hypoestrogenic state with profound ovarian suppression and has a direct endometrial effect with high progestogen levels in the endometrium [150]. There are no conclusive studies examining the differential risk associated with low dose systemic regimens such as progestogen only pill and implants. Furthermore, the protective effect of the other newer combined hormonal contraceptive methods remain to be investigated.

As postmenopausal hormonal replacement therapy

Menopausal HRT is required by millions of women for climacteric symptom management and for the prevention of osteoporosis throughout the world [152]. Studies consistently find a substantial increase in EC incidence with oestrogen alone use [152, 153], which is more marked with prolonged duration of use and persists for several years after treatment discontinuation [154]. As a result, combined oestrogen-progestin therapy is the standard, to counteract the proliferative effects of oestrogen on the endometrium [153]. Results from the Women's Health Initiative study concluded that oestrogen plus progestin use was associated with a 37% reduction in type I EC incidence [152], whilst, oestrogen therapy was found to have little impact on the risk of type II EC [153]. Continuous combined oestrogen-progestin therapy was the only HRT regimen associated with an apparent decline in risk whereas long-term (10 years) sequential regimens were associated with an increased risk of EC [153]. Furthermore, the type of synthetic progestogen does not affect the risk reduction [154]. The use of topical vaginal oestrogen preparations has not shown to significantly increase the risk of EC [154].

As hormonal replacement after endometrial cancer treatment

HRT after treatment of EC remains controversial but studies have specifically looked into this group of patients and shown no significant increase in the risk of recurrence in EC survivors using HRT relative to the control group (OR=0.53; 95% CI, 0.30–0.96) [155]. However, personalising HRT to patient with ER negative EC may be considered to reduce any potential adverse effect.

Progestogens as treatment for hyperplasia

As hyperplasia is due to excess or unopposed oestrogenic effect in the endometrium, prophylactic treatment with high dose oral progestogens such as MPA and local administration with LNG-IUS is used to prevent progression of hyperplasia to EC [156]. Systemic administration of high dose progestogens are associated with an exceptionally poor side effect profile, low compliance and lower remission rates (60%) [157] compared with the LNG-IUS [158, 159]. The local endometrial LNG levels achieved with IUS exceeds that of oral administration by over 1000 folds with higher remission rates (100%) and the additional benefit of a more beneficial side effect profile [157], hence it is the preferable method of delivery. Progestogen treatment using LNG-IUS is also commonly used in women with obesity and other high-risk conditions (e.g. PCOS, tamoxifen treatment) mentioned above as part of a risk reduction strategy.

Progestogens as treatment for endometrial cancer

Synthetic progestogens have been used to treat EC for over 60 years, and they remain to be the main hormonal pharmaceutical agent used in EC. High dose progestogens, in the form of oral MPA and megestrol acetate have been licensed for fertility-sparing treatment of carefully chosen individuals with early stage, low-grade endometrial disease [160]. They are also routinely used as an adjuvant therapy in advanced, metastatic and recurrent ECs as well as in endometrial stromal cell cancers [161]. However, the efficacy of progestogens as an adjuvant treatment in high-risk ECs has been poor or modest at its best [162]. This observed inconsistent response to progestogens in clinical studies that can be explained by the fact that treatment is not tailored according to ECs with PR expression/progestogen responsive.

When considering hormonal modulators, one striking feature specific to EC is that presently, there is no consensus in routinely assessing ECs for their hormone responsiveness (assessing the expression of steroid hormone receptors like in other hormone responsive cancers e.g. breast cancer). Due to the distinct alterations of the steroid receptor expression observed in EC subtypes and with tumour progression (Figure 1), this is an important deficiency in current practise. We have recently proposed a validated scoring system to quantify PR (and other hormone receptor expression) in ECs, as an endeavour to provide means to identify potential responders [25]. Prolonged therapy with progestogens can render the endometrial cells unresponsive to hormone treatment by downregulating most (if not all) ovarian steroid hormone receptors. Considering this complex interplay of hormonal actions in the endometrium, a suitable sequential regimen of hormonal agents (progesterone, followed by anti-oestrogens) [157] and personalising/tailoring treatment according to sequential assessment of the tumour for hormone responsiveness is envisaged to increase the therapeutic potential of endocrine therapy in EC.

Selective progesterone receptor modulators (SPRMs)

Mifepristone is the first SPRM, and has partial agonist/antagonist activity with significant affinity to AR and GR. Therefore, the observed endometrial effect depends on the availability of progesterone, and at least some of the observed effects of mifepristone may be via the AR [163]. Long-term use of high-dose mifepristone thickens the endometrium, although the associated histology shows cystic glandular atrophy with reduction in glandular mitosis. Nevertheless, concerns have been raised regarding a potential trophic effect of SPRMs on the endometrium [164]. Ullipristol, a 2nd generation SPRM, is also licenced to be used for fibroid-associated symptoms and has been reported to have lower endometrial trophic effects. There are reports of hyperplasia without atypia and polyps after 13 weeks of Ullipristol treatment, yet the endometrium reverted to normal, 6 months after treatment [165]. In a small Phase II study, high-dose mifepristone as a single agent therapy in women with either advanced or recurrent EC, revealed very limited stabilisation of the disease, and no partial or complete response [166]. Therefore, until further conclusive data are available, clinicians using high dose prolonged therapy with SPRMs in women need to be aware of possible endometrial changes similar to that occur with tamoxifen [166, 167].

Selective oestrogen receptor modulators (SERMs)

SERMs represent a group of non-steroidal non-hormonal compounds that bind to the ER, each inducing a

distinct set of tissue-specific effects. The agonistic or antagonistic effect of SERMs depend on the availability of oestrogen [168].

Tamoxifen, the first clinically applicable SERM is used as an adjuvant treatment and as a chemoprevention agent in breast cancer. Tamoxifen therapy, is associated with subsequent endometrial proliferation in PM women, with the development of a spectrum of endometrial lesions, ranging from hyperplasia and polyps to invasive carcinomas and sarcomas due to its ER agonist activity in the uterus [169]. The increased risk of EC with tamoxifen use seems to be proportionally related to longer duration, accumulative usage, and increased BMI. A recent opinion paper suggested that premenopausal women treated with tamoxifen require no additional monitoring beyond routine gynaecologic care due to lack of evidence of an increased risk of EC [170].

Conversely, the newer SERMs, such as Raloxifene, Bazedoxifene and Ospemifene, seem to have neutral effects on the endometrium [171–173]. Lasofoxifene, on the other hand, may increase endometrial thickness but the early studies have not confirmed a higher risk of hyperplasia or EC after 5 years of follow-up [174].

Tibolone, a synthetic steroid with oestrogenic, (and some progestogenic and androgenic) properties, is commonly used for climacteric symptoms and prevention of osteoporosis. It has also been recently reported to increase the risk of EC [153], however, a previous RCT suggested that tibolone use has no concerns on endometrial safety [175]. Therefore, further research is necessary to conclude on the effects of SERMs and tibolone on endometrial carcinogenesis.

Antioestrogens

Fulvestrant is a full ERα and ERβ antagonist used as a second line drug for the treatment of breast cancer. Prospective endometrial assessment of breast cancer patients treated with fulvestrant showed a significant decrease in endometrial growth without the development of new endometrial pathologies [176]. Phase II studies have shown modest activity in advanced or recurrent EC patients with ER and/or PR positive tumours [177, 178]. The effectiveness of fulvestrant in EH and fertility-sparing EC management has not yet been assessed.

Aromatase inhibitors (AI)

Third generation AIs (letrozole and anastrozole) are non-steroidal competitive inhibitors of the aromatase enzyme that act to decrease systemic and intra-tumoral oestrogen levels. Prospective endometrial assessment of breast cancer patients treated with AI show no effect on endometrial thickening and possible regression of baseline endometrial proliferation [179]. Further studies also confirm that EH respond to AI, and the efficacy is comparable to progestogens [180–182]. Likewise, regression in response to combined AI and MPA was reported for fertility-sparing low-grade EC, whereas AI monotherapy failed to show any benefit for advanced or recurrent EC patients [183, 184]. Collectively, these results suggest a potential role for AI in early EC stages, requiring further investigation to identify synergistic pathways.

Androgens

Testosterone transdermal patch and subcutaneous preparations are used as part of some HRT regimens, particularly in women suffering from symptoms of androgen deficiency, such as low sexual desire and well-being. Current data does not indicate any adverse endometrial, cardiovascular or breast effects with transdermal preparations, although data on long-term risks and benefits of testosterone therapy after the diagnosis of EC are lacking [185, 186]. Endometrial atrophy is the common finding in transgender women on supraphysiological doses of testosterone (Testoviron Depot), instead of hyperplasia [187].

Oral and transvaginal dehydroepiandrosterone (DHEA) has been used for controlling PM symptoms. Although significant benefit remains to be determined, reports have shown a persistent atrophic phenotype in PM endometria after 52 weeks of this treatment [188].

Local danazol therapy which is used to improve symptoms associated with endometriosis has shown to act on endometrial epithelial and stromal cells directly through AR (also binds PR) by reducing cell survival [189] and cell proliferation [190, 191]. Data from a murine model shows a protective effect of long-term danazol against oestrogen induced carcinogenesis [192]. Likewise, clinical trials have also confirmed the effectiveness of danazol in the management of EH with and without cytological atypia with very low incidence of recurrence [193, 194]. By contrast, minimal response to danazol was reported in advanced, recurrent or persistent EC [195], yet sample size and lack of data on AR expression status limits the results of this particular trial.

Cyproterone acetate, an AR antagonist, has been used in HRT combined with cyclic oestradiol. Studies have shown an increased risk of EC for patients on this regimen compared with non-users [153]. Therefore, androgens are

likely to play an important role in the pathogenesis of EC and the emerging evidence suggests that pharmaceutical agents with AR modulatory activity may have a potential therapeutic effect. Further studies on the effects of androgen on endometrial cell proliferation and function are therefore needed to identify the potential therapeutic strategies.

GnRH agonists and antagonists

Due to differences in the intracellular signalling cascades, the effect observed with GnRH agonists and antagonists is tissue-specific [196–198]. The clinical effects of the GnRH analogues seen after sustained administration, leads to desensitisation of receptors, suppression of gonadotropin secretion and finally inhibits the gonadal steroid synthesis.

The GnRH-R are currently being targeted successfully in the treatment of many hormonal dependent tumours using GnRH agonists and antagonists. Preparations with pharmacological properties of GnRH agonists, antagonists, GnRH agonist-based cytotoxic hybrids, GnRH-R targeted nanoparticles delivering anti-cancer medications act through GnRH-R are being explored in this respect. GnRH agonists show promise in treating advanced, and metastatic prostatic and breast cancer treatment. Unlike the 1st and 2nd generation antagonists with limited solubility and anaphylactic reactions due to histamine release [199], the currently available 3rd and 4th generation antagonists like cetrorelix and ganirelix have a more favourable side effect profile and for example, have immensely improved prostate cancer therapy. Although some in vitro studies have suggested a possible anti-tumour effect with GnRH analogues in EC, there are no clinical studies reported to date evaluating their efficacy in EC. Therefore, further research is urgently needed to assess if GnRH has a therapeutic role in EC.

Summary/Conclusion

Hormones intricately influence the carcinogenesis process in endometrium. Whilst oestrogen is the most conspicuous driver of this, many other hormones also appear to play an important role. Most known risk factors for EC are associated with alteration of the endometrial hormonal milieu, with many routinely used pharmacological hormonal agents impinging on the endometrial carcinogenesis process. Routine assessment of ECs for their hormone responsiveness (assessing the expression of steroid hormone receptors as in other hormone responsive cancers), using a validated scoring system, personalisation of treatment according to sequential assessment of the tumours and further evaluation of interplay between all hormones in the endometrium are envisaged to unlock novel, therapeutic avenues, based on hormones, for this increasingly common malignancy.

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National and International conference presentations

Tempest N, *Baker AM, Jensen M, Wright N, Hapangama D*. 3D Architecture of endometrial glands in relation to stem cell organisation. Society for Gynaecologic Investigation, San Diego, March 2018. (Oral presentation - **SRI President's Plenary Award**)

Tempest N*, Baker AM, Wright N, Hapangama D*. LGR5, the proposed universal stem cell marker is hormonally regulated in the human endometrium. RCOG National Training Conference, Leeds, Nov 2017. (Poster presentation)

Tempest N, *Baker AM, Jensen M, Wright N, Hapangama D*. **3D Architecture of endometrial glands in relation to stem cell organisation.** North of England Obstetrics and Gynaecology society, Liverpool, Oct 2017. (Oral presentation – **Second prize**)

Tempest N, *Baker AM, Wright N, Hapangama D.* LGR5 is expressed by human endometrial epithelial cells and regulated by progesterone.

Society for Gynaecologic Investigation, Florida, March 2017. (Oral presentation)

Tempest N*, Hapangama D.* LGR5 is expressed by human endometrial epithelial cells and regulated by progesterone. North West Cancer Research, Liverpool, March 2017. (Poster presentation)

Tempest N*, Hapangama D.* LGR5 is expressed by human endometrial epithelial cells and regulated by progesterone. RCOG Academic meeting, London, Feb 2017. (Poster presentation)

Tempest N*, Hapangama D.* LGR5 is expressed by human endometrial epithelial cells and regulated by progesterone. National RCOG Trainee Conference, Newcastle, Nov 2016. (Poster presentation)

Tempest N, *Hapangama D.* LGR5 is expressed by human endometrial epithelial cells and regulated by progesterone. Gordon Research Conference, Hong Kong, Aug 2016. (Poster presentation)